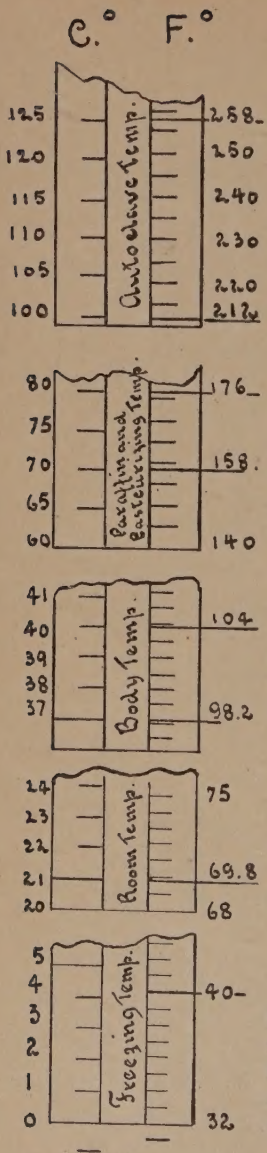


Equivalent Fahrenheit and Centigrade tables for the temperatures in common use in laboratories:

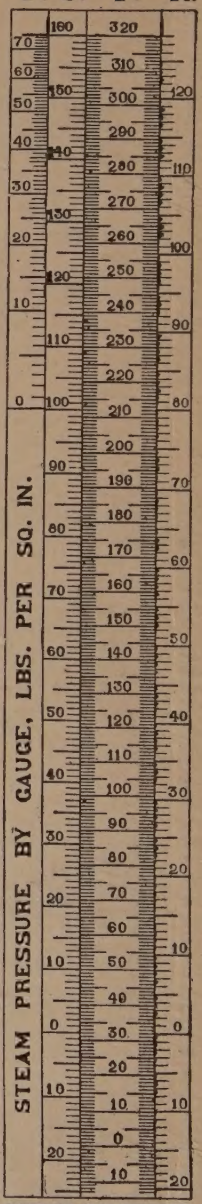
1. Those for sterilization of dressings and media. Also for certain disinfection of spore-bearing bacterial contamination (autoclave temperatures).

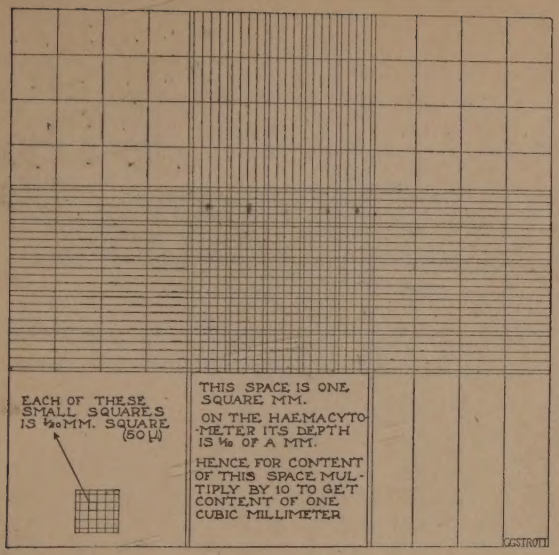
2. Those for pasteurization and sterilization of bacterial vaccines. Also for paraffin bath (pasteurizing temperatures).

3. Those for growing important pathogenic organisms (body temperatures).

4. Those for culturing gelatin (melting point, 25°C.) as in water work (room temperatures).

5. Those employed in preserving biological products and post-mortem material; also in centrifuging experiments to separate complement and amboceptor (freezing temperatures).




EACH OF THESE SMALL SQUARES IS 1/20 MM. SQUARE (50 μ)
THIS SPACE IS ONE SQUARE MM.
ON THE HAEMACYTOMETER ITS DEPTH IS 1/10 OF A MM.
HENCE FOR CONTENT OF THIS SPACE MULTIPLY BY 10 TO GET CONTENT OF ONE CUBIC MILLIMETER
GSTROTH

The Improved Neubauer Ruling. Explanation of the Squares.—In the first place, we have the square which encloses the entire ruled surface. This is made up of nine squares, each 1 mm. square. These are the squares to use in connection with leukocyte counts with the white pipette. They may be termed the large squares.

The central square millimeter is divided into 400 small squares each of 1/400 square millimeter area. (The improvement in this ruling over that of the Thoma and the original Neubauer consists of the division of these small squares into 25 groups of 16 each by a "split" fifth line.) These small squares are the ones used in connection with erythrocyte counts with the red pipette.

The unit in estimating the leukocyte or red cell content of blood is the cubic millimeter which is 1/1000 of a cubic centimeter.

In making a leukocyte count we usually take the white pipette, which has the mark 11 just above the bulb, and draw up the blood to 0.5 and then with suction we fill the pipette to the 11 mark with the diluting fluid for which a 0.5% solution of glacial acetic acid in water is most satisfactory. This gives a dilution of 1–20.

Counting with the 2/3 inch objective all of the highly refractile dots representing leukocytes in one of the 1-mm. squares at either of the four corners we note the number and mentally multiply by 20 (the number of times the blood was diluted). As the depth of the diluted blood between the ruled surface of the haemacytometer slide and the under surface of the cover glass is only 0.1 of a millimeter, we multiply the figure as above obtained by 10 to get the number of cells in a 1–20 dilution of blood in a space of one cubic millimeter.

Example: Counted 90 leukocytes; $90 \times 20 = 1800$; $1800 \times 10 = 18{,}000$; equals number of leukocytes in one cubic millimeter of blood.

For red counts we use the red count pipette which has the 101 mark just above the bulb. Taking up blood to 0.5 we draw up the diluting fluid to 101. This gives a dilution of 1–200. Counting the red cells in five of the aggregations of 16 small squares (1/20 mm.) thus having counted 80 small squares we have counted 1/50 of the total number of small squares in a cubic millimeter, there being 4000 small squares in a cubic millimeter. Consequently the number of red cells in 80 small squares multiplied by 50 and then by the dilution of 200 gives the number of red cells in one cubic millimeter of the blood examined.

It is well to make a second preparation and record the average of the two counts.

PRACTICAL BACTERIOLOGY, BLOOD WORK AND ANIMAL PARASITOLOGY

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INCLUDING

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A COMPENDIUM FOR INTERNISTS

BY

E. R. STITT, A.B., PH.G., M.D., SC.D., LL.D.

REAR ADMIRAL, MEDICAL CORPS, AND SURGEON GENERAL, U. S. NAVY; GRADUATE, LONDON SCHOOL OF TROPICAL MEDICINE; PRESIDENT NATIONAL BOARD OF MEDICAL EXAMINERS; MEMBER FEDERAL BOARD OF HOSPITALIZATION; PRESIDENT BOARD OF VISITORS, ST. ELIZABETH'S HOSPITAL; MEMBER EXECUTIVE AND CENTRAL COMMITTEES, AMERICAN RED CROSS. FORMERLY: COMMANDING OFFICER AND HEAD OF DEPARTMENT OF TROPICAL MEDICINE, U. S. NAVAL MEDICAL SCHOOL; PROFESSOR OF TROPICAL MEDICINE, GEORGETOWN UNIVERSITY; PROFESSOR OF TROPICAL MEDICINE, GEORGE WASHINGTON UNIVERSITY; LECTURER IN TROPICAL MEDICINE, JEFFERSON MEDICAL COLLEGE; ASSOCIATE PROFESSOR OF MEDICAL ZOOLOGY, UNIVERSITY OF THE PHILIPPINES

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PREFACE TO EIGHTH EDITION

When it became evident that a new edition of this laboratory manual of internal medicine would soon have to be prepared, I sought through eliminating methods of technique and subject matter which had been made of less value by recent advances in clinical pathology, and through more extensive use of small-type paragraphs, to reduce the size of this book by approximately one hundred pages. It soon became evident, however, that the important additions to our knowledge of clinical pathology in the past four years would make the plan of a "pocket manual" impossible; indeed, it proved necessary, notwithstanding extensive deletion and employment of smaller type, to increase the size of the book by about the number of pages it had been planned to reduce it.

With the wide acceptance of the new bacterial nomenclature, as presented in the second edition of Bergey's Determinative Bacteriology, it seemed necessary to consider the advisability of changing the names of bacteria of medical importance. Recognizing the conservatism of many medical men I have decided to retain the previously accepted names in the descriptive matter and clinical notes of the bacteria important in human pathology; but in the "Keys," preceding the consideration of each group of bacteria, to make use of the nomenclature approved by the Society of American Bacteriologists. In the Keys and in the subject matter both the old and the new names are given,—in the Keys the old name in parentheses following the new one, and in the subject matter the new one following the long-accepted designation.

The importance of consultation with members of the dental profession in the conduct of periodic health examinations and the bearing of dental pathology on many diseases have made it seem necessary to outline the methods of medical and dental cooperation practiced in the diagnostic clinic of the U. S. Naval Medical School and the U. S. Naval Dental School. Chapter XXVIII—"Diagnosis of Infections of the Teeth," was prepared with the assistance of Comdr. Walter A. Bloedorn, Consulting Internist of the Naval Medical School, and Lt. H. R. Delaney, of the Naval Dental Corps. In the preparation of

this chapter we have consulted freely the authoritative manual of Professor Appleton, "Bacterial Infection."

Due to the relative simplicity of the Kahn test, the stability of the antigen it uses, and its high specificity, the test was made standard in the Naval service in December, 1925. Experience with the test during the period that has elapsed has been conspicuously satisfactory,—to such a degree that I feel justified in presenting it in this edition as the method of choice for most laboratory workers. Accordingly, I have included a comprehensive description of the method and variations, kindly prepared by Dr. Kahn, to whom I desire here to express my thanks. In the large base-hospital laboratories the Wassermann test, for the present, is made along with the Kahn test. It will be remembered that in the seventh edition Professor Noguchi prepared the text dealing with the complement-fixation test bearing his name.

Originally this little manual was prepared to serve as a text book in clinical pathology for the student officers at the Naval Medical School, and in conducting each revision I have been guided by my experience in teaching and by the criticisms of the students. Having found such experience of great value in deciding as to changes or additions, and in order to continue to avail myself of it, I have requested the several instructors at the Naval Medical School to make suggestions concerning the presentation of the subjects taught by them.

My chief indebtedness is to Lt.-Comdr. John Harper, the Director of the laboratories of the Naval Medical School. In the section on bacteriology Dr. Harper has cooperated with Dr. Houghton, the Instructor in Bacteriology, who prepared the first outline of the changes, and to them is due the substitution of the new nomenclature in the bacteriological keys as well as the addition to the text of many recent advances in bacteriological knowledge. Particular attention is invited to the many additions and changes under the streptococcus group—particularly as bearing on scarlet fever, measles, erysipelas and poliomyelitis.

Some of the most important changes of this edition have been in Part II "Study of the Blood." Chapter XIII has been brought up to date by Commander Bloedorn and Lieutenant Commander Houghton, many of the older methods of cell counting and other items of technique having been replaced by more recent procedures. The sedimentation test is extensively discussed in this chapter. In Chapter XIV Dr. Bloedorn has added a new section—the haemorrhagic diseases, and also a discussion of sickle-cell anaemia and glandular fever.

In Part III the changes in zoological nomenclature have been almost as great as with the bacteria. Dr. Harper, with a few exceptions (e.g. malaria and proflagellata), has followed the key catalogues of Stiles and Hassall and I take this occasion to thank Prof. Charles Wardwell Stiles of the Hygienic Laboratory for his unvaried willingness to give us his opinion on disputed matters of nomenclature.

In the section on the Protozoa we have considered Wenyon's Protozoology as the most authoritative source to consult in the subject matter.

Dr. Freeman, the Director of the laboratories of St. Elizabeth's Hospital, has kindly prepared the section on neurological staining methods in the section on tissue pathology.

An entirely new section on liver functioning tests has been prepared by Lt. W. W. Hall, the Instructor in medical chemistry, who has also revised the text dealing with clinical chemistry, aided in the latter task by suggestions from Commander Bunker, who revised the section on chemistry of the seventh edition.

The section on nutrition in health and in disease has been revised by Commander Phelps, head of the department of Preventive Medicine and Hygiene, who only shortly before had published a detailed study of the Navy Ration in the light of modern knowledge.

The section on disinfectants and insecticides has been brought up to date by Chief Pharmacist Setterstrom, one of our chemical specialists.

We have been fortunate in having had also the advice and assistance of Dr. McCoy, Director of the Hygienic Laboratory, and his colleagues in the revision of various sections of the book. In particular has this help been valuable in the revision of the subject matter of smallpox vaccination by Dr. Leake, scarlet-fever immunity by Dr. Dyer, and in suggestions as to bacteriological changes by Miss A. C. Evans and Mrs. E. M. A. Enlows.

In almost every chapter of the book will be found changes and additions suggested by Capt. H. W. Smith, of the Naval Medical Corps. Dr. Smith edited the seventh edition but has found many changes necessary to meet advances in medical science taking place in the past four years.

In equal degree I have to thank Capt. C. S. Butler, the Commanding Officer of the Naval Medical School, for numerous notes of changes and additions recommended. Many of these notes are the fruit of Dr. Butler's experiences while serving as Chief Health Officer of Haiti.

Along with Dr. Harper I am particularly indebted to Lieutenant Soukup, of the Naval Medical Corps, who has assisted Dr. Harper in the revision and in the proof-reading, as well as in the preparation of the index.

I am also indebted to Lieutenant Commander Peterson, who assisted Dr. Smith in the revision of the seventh edition, and to Comdr. G. F. Clark, for many notes and suggestions as to changes.

E. R. Stitt.

Washington, D. C.,
1927.

EXTRACTS FROM PREFACES OF PREVIOUS EDITIONS

While a member of the Naval Examining Board and examiner in bacteriology and clinical microscopy, I have had an opportunity to judge of the qualifications of several hundred graduates of the various medical schools of the country from the standpoint of practical application in the laboratory of that which they had learned as under-graduates.

More particularly I have made it a point to ascertain from the successful candidates, while under instruction at the Naval Medical School, the features of their laboratory courses which had seemed to them most practical, the methods being subsequently tested in our own class work.

As a result I have endeavored to incorporate in this manual methods which have been submitted to the criticism of postgraduate students from all the leading medical schools of the country, and which have been considered by them adapted to the requirements of practical, speedy, and satisfactory clinical laboratory diagnosis.

In practical work, organisms can be separated culturally only by the use of Keys and for this reason Keys are given at the beginning of each division of bacteria. These enable one quickly to place the organism isolated in its respective group.

The giving of the cultural characteristics in a systematic tabulated Key gives space in the notes for presenting the salient points in the pathological and epidemiological aspects of each organism.

While there is no difference between the laboratory requirements of medical work in the tropics and that in temperate climates, unless by reason of such measures of diagnosis being indispensable in the tropics, it has, however, been my endeavor to treat every tropical question, whether in blood work, bacteriology, or animal parasitology, in a more complete way than is usual in manuals of this character. Therefore it is believed that this little book will be of great service to the laboratory worker in the tropics.

In animal parasitology the system of arranging tables, showing the families, genera, etc., in which each species belongs will, it is believed, greatly simplify the matter of classification for the medical student. The points given under each parasite are believed to be practical ones. When a parasite has only been reported for man two or three times, very little space is given to it.

Part IV summarizes the various infections which may be found in different organs or excretions of the body and embraces both bacterial and animal parasites. Practical methods for examining material are also given.

The chapter on Immunity, in which the theoretical side is immediately illustrated by the practical application, will tend to simplify this bug-bear of the medical student.

The illustrations have been selected with a view to bringing out points which are difficult to state briefly in the text, and furthermore they have been grouped together so that comparison of similar parasites is possible without turning from page to page. (*From first edition.*)

As noted in previous prefaces, this manual represents the notes of a course along the laboratory side of internal medicine which has seemed to the author practical and concise.

It will be noted that I have combined notes on points of clinical importance with laboratory details. Such a presentation of the subject would appear of more value to the student of internal medicine than the bare laboratory technic.

In view of the enormous advances in internal medicine since the appearance of the last edition it has been very difficult to incorporate new material and at the same time retain the pocket manual feature. This has been done, however, by the greater use of paragraphs of smaller type separating those printed in larger type, a plan which adds to the accessibility of the contents as well as lessening the number of pages.

To further facilitate quick reference numerous additional bold type headings of paragraphs have been introduced.

A new chapter, dealing with diseases of doubtful or only recently determined etiology has been added to Part IV. In this will be found discussions of the vitamin theory in beriberi and pellagra as well as recent findings in connection with such diseases as typhus fever, Oroya fever, verruga, rat bite fever, spotted fever of the Rocky Mountains and sprue. (*From fourth edition.*)

The original plan of a laboratory manual of internal medicine in which clinical notes are presented has been adhered to in the present edition. Such a method emphasizes the necessity for utilizing equally every diagnostic aid in the study of a case.

At the end of the appendix a section which it is believed will be of great help to those doing laboratory work only occasionally has been inserted. This section presents the laboratory procedures indicated in the investigation of the more important diseases and is arranged alphabetically. (*From sixth edition.*)

The importance of dietetic faults in the production of disease and in lowering the resistance of the body to bacterial invasion has become so clearly recognized that I have felt it necessary to insert a summary of the subject of nutrition for the convenience of those who may not have ready access to recent literature dealing with this most rapidly advancing science. Its introduction, I feel, needs no apology since studies in nutrition hold in prospect results of greater benefit to mankind than any other line of current investigation pertaining to public health.

Among others, whose courtesies I take pleasure in acknowledging, are Dr. Paul Bartsch, for placing at my disposal the results of his studies in the classification of mollusks; Dr. Carroll Fox for two of the illustrations of fleas; Messrs. Howard, Dyar and Knab for much of the material presented in the chapter on "Mosquitoes," and the Carnegie Institution for permission to reproduce illustrations. (*From seventh edition.*)

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BACTERIOLOGY, BLOOD WORK AND ANIMAL PARASITOLOGY

PART I

BACTERIOLOGY

CHAPTER I

APPARATUS

The Microscope

The most important piece of apparatus for the laboratory worker is the microscope. Very satisfactory microscopes can be purchased in this country. It is impossible to do good microscopical work unless the microscope gives and continues to give good definition and the working parts remain firm.

Folding microscope stands are now made which are perfectly satisfactory. Such instruments, however, have the advantage only of occupying less space in a case so that unless the question of compactness is involved, as in an outfit for the military services or for a microscopist who travels about a great deal, the ordinary rigid horseshoe base is to be preferred.

A mechanical stage is almost a necessity in connection with blood work and its use is advantageous in bacterial preparations. For the study of tissue sections, however, the moving of the slide with fingers is preferable, and therefore, the mechanical stage should be capable of ready attachment or removal. For the examination of colonies growing in Petri dishes we also use the stage unencumbered with the mechanical stage. A triple or quadruple nose-piece, the choice depending on the number of objectives commonly used, is also indispensable.

One should always use a magnifying glass or, better, a lens in a tripod or an achromatic triplet in the preliminary study of microscopic objects. A dissecting microscope is even better for this purpose and is very useful in dissecting mosquitoes, etc. In fact the dissecting microscope is almost essential in the examination of helminthological and entomological specimens. Of particular value is a stage forceps for orienting insects, especially mosquitoes, when examined on the stage of either a compound or simple microscope.

The following precautions should be observed to prevent injury to the microscope:

1. If the fine adjustment works through the arm of the microscope, always grasp the instrument by the pillar which supports the stage. In those microscopes, however, which are not constructed in this way the arm has a handle portion made to serve in lifting the instrument.

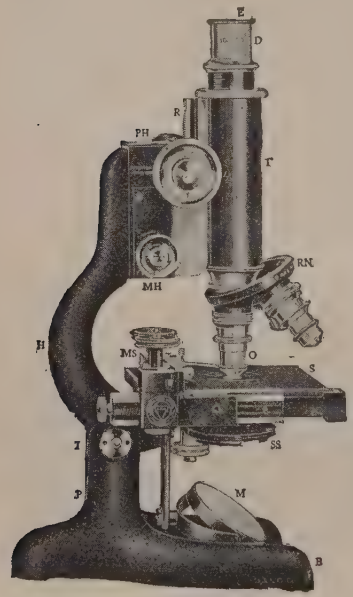


FIG. 1.—Parts of microscope. *E*, Eye-piece. *D*, Drawtube. *R*, Rack (coarse adjustment). *PH*, Pinion head. *T*, Body tube. *MH*, Micrometer head (fine adjustment). *RN*, Revolving nose-piece. *O*, Objectives. *MS*, Mechanical stage. *S*, Stage. *H*, Handle, a part of the arm. *SS*, Substage. *I*, Inclination joint. *P*, Pillar. *M*, Mirror. *B*, Base.

2. Always keep the microscope in its case or covered with a bell jar when not in use in order to keep away the dust. A piece of black cloth supported on a wire lamp-shade frame makes a most convenient protecting covering.

3. Care should be observed to keep all parts of the microscope from coming in contact with acids, alkalies, chloroform, xylol and alcohol.

4. Always use Japanese lens paper in wiping off the dust from dry objectives or the immersion oil from the 2-mm. one. Should one neglect to wipe off the oil from an oil-immersion objective, the dried oil can be removed by wiping with lens paper moistened with a drop of xylol, but the cleaning should be done as rapidly as possible, with a final wiping off with dry lens paper, to avoid damage by the xylol to the setting of the lenses. Throw lens paper away after using it once.

5. Lenses are very liable to deteriorate in the tropics. One should be careful to protect his instrument from the direct light of the tropical sun.

6. If any oil is used on the mechanical parts for lubrication, all excess should be wiped off to avoid the catching of dust or gritty particles.

Objectives.—To meet the demands of clinical microscopy there should be three objectives, preferably a 16-mm. (⅔-inch), a 4-mm. (⅙-inch) and a 2-mm. (1/12-inch) homogeneous oil-immersion. Some workers prefer a ⅕-inch objective to the ⅙-inch.

A dust-proof quadruple nose-piece with four objectives will be found a great convenience (in addition to the ⅔-inch and 1/12-inch objectives, a ¼-inch for urine and blood counting, with a ⅛-inch for examining hanging-drop preparations and for

quick examination of blood smears). An apochromatic objective costs about three times as much as an achromatic one and, except in photographic work has little if any advantage.

Objectives are usually designated by their equivalent focal distance. It is important to remember that the equivalent focal distance does not represent the working distance of an objective, by which is meant the distance from the upper surface of the cover glass to the lower surface of the objective. Thus a ¼-inch objective may have to be approached to the object so that the distance intervening may be only $^1/_6$-inch or even less. This explains the frequent inability to focus an object when a high-power dry objective ($^1/_6$-inch or ⅛-inch) is used with a rather thick cover glass—the objective possibly having a short working distance, so that the thickness of the cover glass does not allow of any free working distance.

In addition to being marked with the equivalent focal distance in mm., the Spencer objectives are also marked with the N.A. in mm. and the magnification. The Bausch and Lomb objectives are marked only with the equivalent focal distance and the N.A. in mm.

The Zeiss AA is a 17-mm. objective, and the Leitz No. 3, an 18-mm. or ¾-inch one. The Zeiss D is about 4.2 mm. and the Leitz No. 6, 4.4 mm. or $^1/_6$-inch.

Oil Immersion.—In using the oil-immersion objective, place a drop of cedar oil on the preparation; then, using the coarse adjustment, lower the objective through the oil, stopping just before contact with the slide or cover glass is made, or as soon as actual contact is felt. Note that if the fine adjustment is used contact cannot be felt and, as a result, the lens may be injured. The movement of the lens should be controlled by the eye at a level with the stage. Once the position described is reached, the lens then may be slowly elevated until the object is observed to be in focus. It saves time and disappointment to make a preliminary examination of a preparation with a low power ($^2/_3$-inch) before employing a higher power; in this way we locate or center a suitable field for study.

Numerical Aperture.—It will be observed that objectives frequently have their numerical aperture marked on them. This is expressed by the letters N.A. From a practical standpoint this gives the relative proportion of the rays which proceeding from an object can enter the lens of the objective and form the image. Of course, the greater the number of rays, the greater the N.A., the better the definition, and consequently the better the objective. Immersion oil, having the same index of refraction (1.52) as glass, does not deflect rays coming from the object and so prevent their entering the objective, as would be the case if we used a dry objective with an intervening air space. In this case a portion of the rays would be turned aside by the difference in the refractive index of air. As a rule, a higher N.A. is gained at a sacrifice of depth of focus and also of working distance. Hence, in blood counting with the haemacytometer, the cover glass being comparatively thick, it may happen that with a $^1/_6$-inch of high numerical aperture there may not be sufficient working

distance to bring the blood cells into focus, which could be done with an objective of lower numerical aperture. Consequently, we must always consider the matter of working distance as well as that of numerical aperture. The skill of the optician, however, can obviate this defect in an objective of high numerical aperture so that it may combine the qualities of perfect definition with sufficient working distance.

Oculars.—These are as a rule numbered according to the amount they increase the magnification given by the objective; thus a No. 2 increases the magnification, given by the objective alone, twice; a No. 8, eight times.

The Spencer, Bausch and Lomb, and Zeiss oculars are marked in this manner. Oculars increasing the magnification given by the objective 5 and 10 times will be found to fulfill all ordinary requirements.

Some oculars are classified according to their equivalent focal distance and are referred to as ½-inch, 1-inch, and 2-inch oculars.

A 1-inch or 25-mm. ocular magnifies the magnification produced by the objective about ten times while a 2-inch or 50-mm. one increases the magnification of the objective five times.

A Leitz Huyghenian eyepiece No. 0 has an equivalent focal length of 62.5 mm. (2½ in.) and magnifies four times. The Nos. 1, 2, 3, 4 and 5 have an equivalent focal length of 50 mm. (2 in.), 41.65 mm. (1⅔ in.), 31.25 mm. (1¼ in.), 25 mm. (1 in.) and 20.85 mm. (⅘ in.) respectively, which give eyepiece magnifications of 5, 6, 8, 10 and 12 times.

The oculars in common use are known as negative or Huyghenian oculars, by which is meant an ocular in which the lower lens (collective) assists in forming the real inverted image which is focused at the level of the diaphragm within the ocular. When using a disc micrometer, it is supported by this diaphragm, and the outlines of the image are cut by the rulings on the glass disc, and so we are enabled to measure the size of the object examined. The measurement of various bacteria, blood cells, and parasites is exceedingly simple and assists greatly in the study of bacteria, and is indispensable in work in animal parasitology. When an ocular is termed positive, it refers to an ocular which acts as a simple microscope in magnifying the image, the image being formed entirely by the objective and located below the ocular. By fixing one end of a hair on the rim of the diaphragm inside the ocular with a minute drop of balsam one has a satisfactory pointer to locate any particular cell in the microscopic field.

In order to get full advantage from apochromatic objectives, especially in photomicrography, it is necessary to employ with them so-called compensating oculars, which are designed to compensate for and correct the residual color defects in the extra-axial portion of the visual field. These oculars are useful with ordinary achromatic objectives also.

Tube Length.—The tube length is measured from the eye lens of the ocular to the end of the tube into which the objective is screwed. If a triple nose-piece is used the tube length must be shortened accord-

ingly. As a rule objectives are corrected to use with a tube length of 160 mm.

Instrument makers generally specify the thickness of cover glass to be used with a certain tube length, but for all practical purposes it will be found satisfactory to use No. 1 (very thin) cover glasses exclusively. The principal objection to these is that they are more fragile than the No. 2, but with a little practice in cleaning cover glasses this is negligible. Immersion lenses are less affected than dry lenses by the question of a certain thickness of cover glasses for a certain tube length.

One of the most frequent causes of the crushing of microscopical objects and the overlying cover glass or, what is far more important, the breaking of the cover glass of a hanging-drop preparation and consequent risk of infection is the attempt to focus with the fine adjustment.

Focusing.—It should be an invariable rule for the worker to use the coarse adjustment to bring his objective practically into contact with the upper surface of the cover glass having the eye on a level with the stage, then, still using the coarse adjustment (rack and pinion), slowly to elevate the objective, but now looking through the eye-piece. In other words, obtain focus with the coarse adjustment and maintain it with the fine adjustment (micrometer screw). The fine adjustment should only be used after the focus is obtained. When using a low power objective (⅔-inch), as in examining a worm or insect, I use only the coarse adjustment.

Micrometry

In the examination of blood and faeces preparations, especially when the identification of animal parasites is in question, there is nothing that assists more than a knowledge of the measurements of the object studied. The making of such measurements microscopically is termed micrometry.

Micrometry is also important in bacteriology and cytodiagnosis as well as in animal parasitology.

The most practical way of making these measurements is with an ocular micrometer. These can be bought separately, or a glass disc (disc micrometer) with lines ruled on it can be dropped into the ocular to rest on the diaphragm inside. The ruled surface of this glass diaphragm should be placed downward. As was stated in connection with the microscope, the image of the object is formed at the level of the diaphragm rim inside the ocular, consequently the lines of the image cut those of the lines ruled on the glass in the ocular Once having standardized the value of the spaces of the ocular micrometer for each different objective, always using the prescribed tube length, all that is necessary subsequently in measuring is to count the number of lines or spaces which the image of the object fills and then, knowing the value of each space for that objective, to multiply the number of spaces by the value of a single space.

The Micron.—The unit in micrometry is the micron. This is usually written μ and is the $\frac{1}{1000}$ part of a millimeter. There are 1000 microns in a millimeter.

The Stage Micrometer.—To standardize an ocular micrometer, it is possibly more accurate to use a regular stage micrometer scale with ruled lines separated from

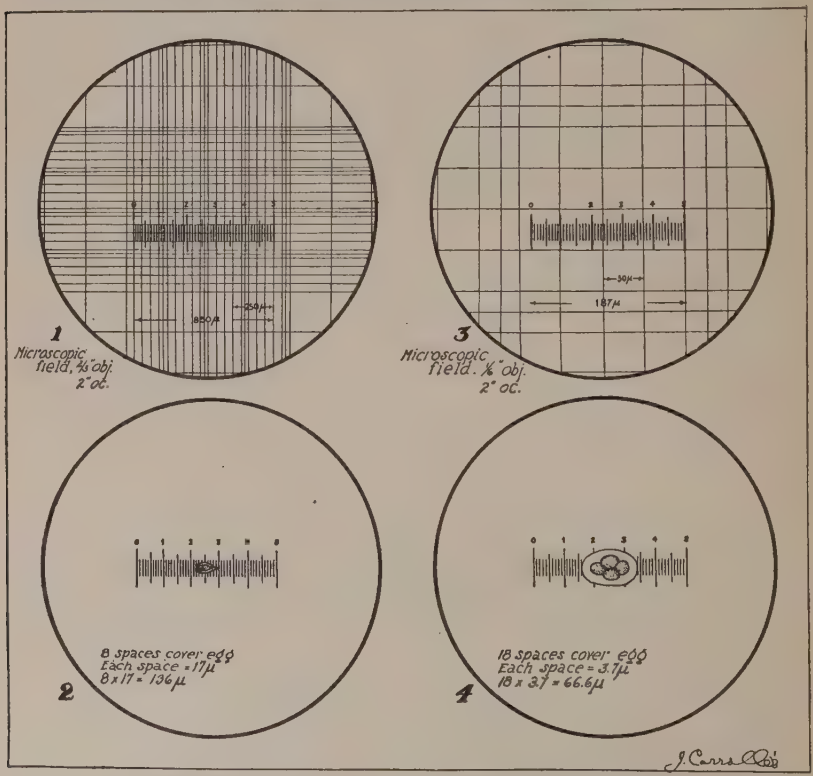


FIG. 2.—1. Fifty lines of ocular micrometer covering 17 small squares paces (50μ each) of haemacytometer. Each ocular micrometer space = 17μ. 2. Schistosome egg fills 8 spaces of ocular micrometer. Egg is 136μ long. 3. Fifty lines of ocular micrometer covering 3.75 small square spaces. Each space = 3.7μ. 4. Hookworm egg fills 18 ocular micrometer spaces. Egg is 66.6μ long.

one another by $\frac{1}{10}$ mm. (100μ). Some of these $\frac{1}{10}$ mm. spaces are again ruled with 10 lines giving spaces which are only $\frac{1}{100}$ mm. (10μ) apart. As one may not have a micrometer scale similar to that just described, but always has a haemacytometer, it is better to use such a scale for standardizing the ocular micrometer. In any system of ruling of the haemacytometer, whether it be the Thoma-Zeiss, Türck or Neubauer one, we have in the central ruling of crossed lines small squares which are

used for counting red cells. These are in groups of 16 and each one is $\frac{1}{20}$ mm. or 50μ square.

Ocular Micrometer.—The ocular micrometer is usually ruled with 50 to 100 lines or spaces, separated by longer lines into groups of 5 and 10.

Having focused the ruling of the haemacytometer we note the number of small squares covered by the 50 or 100 ruled lines of the ocular micrometer and multiply the number of squares so overlaid by 50, which gives the micron value of the entire ocular micrometer ruled space. To obtain the value of each space divide by 50 or 100 according to the number of lines of the ocular micrometer. Having standardized the ocular micrometer for each objective we make a note of such values and need not again repeat the standardization. To measure the egg of an intestinal parasite in faeces we simply focus on the egg and note the number of spaces covering it and multiply this number of spaces by the value in microns of the space for the objective used. I always keep the micrometer disc in the 2-inch ocular so that it is always ready for instant use. As a rule clinical microscopists use the 1-inch ocular as a routine one so that the ocular chiefly used is free of any interfering lines. There is a very slight interference with the view of the microscopic field, when using an ocular micrometer.

The tube length which is used at the time of standardizing must always be adhered to in subsequent measurements.

Filar Micrometer.—The most accurate instrument for measuring is the filar micrometer but it is expensive. Measurements can also be made with the camera lucida, but it takes considerable time to make the adjustments necessary, so that it is not convenient. With an ocular micrometer one can make measurements of blood cells, amoebæ, etc., in a few seconds—it being only necessary to slip in the ocular micrometer.

Rule for determining the magnifying power of microscopic lenses: Measure the diameter of the lens of the objective in inches—the approximate equivalent focal distance is about twice the diameter. Dividing 10 by the equivalent focal distance gives the magnifying power of the lens. This should be multiplied by the number of times the ocular magnifies. Example: The diameter of the lens of the objective was found to measure $\frac{1}{2}$ inch, the focal distance would then be about 1 inch. Dividing 10 by 1 we have 10 as the magnifying power of the lens of the objective. If we were using a No. 4 ocular, the magnifying power would be approximately forty.

Practical Points in the Use of the Microscope.—An important matter in the use of the microscope is to get all the details possible with a low power before using a higher power. This, of course, does not apply to a bacterial preparation where it is necessary to use a $\frac{1}{12}$-inch or a high-power dry lens.

It is well, however, in a bacterial or blood preparation first to examine the smear with the ⅔-inch objective in order to determine suitable areas for examination with the oil-immersion objective. With tissue sections it is not only advisable to begin the study with the lowest power, but even an examination with the unaided eye or with a magnifying glass, before using the microscope, will give a surprising amount of information.

Position.—While some workers prefer to use the microscope with the body tube inclined by the inclination joint yet one gets just as good results by keeping the tube perpendicular and it is better to accustom one's self to such a position, because it is necessitated when we work with fluid mounts.

Care of the oil immersion.—After using the oil-immersion objective the lens should be wiped clean. Dried oil on a lens often causes the lens to be considered defective. Accidental contact of the dry objectives with oil is not uncommon and should always be thought of when satisfactory optical effects are not obtainable. In depositing the drop of immersion oil on the slide bubbles are at times formed which make it almost impossible to use the 1/12-inch objective. Under such circumstances I either prick the bubbles or wipe off the oil and deposit a drop anew.

The eye.—It is advisable to cultivate the use of both eyes in doing microscopical work. When using one eye the other should be kept open with accommodation relaxed, since squinting of the unemployed eye causes fatigue. A strip of cardboard 4 or 5 inches long, with an opening to fit over the tube of the microscope, leaving the other end to block the vision of the unused eye, will prevent the strain. This apparatus can be purchased in vulcanite. Binocular microscopes are now made which are entirely satisfactory, though expensive.

Warm stages.—A warm stage for the study of living protozoa may be extemporized by taking a piece of copper, about the size of the stage, with a strip projecting out anteriorly for 5 or 6 inches. The under surface of the plate is covered with flannel and a hole about 1 inch in diameter cut out of the center. The proper amount of heat is applied by a flame impinging on the tongue-like projection of the copper plate.

At present there are electrically heated warm stages, connected with the desk socket by a wire and plug, which are most convenient. In fact they are as satisfactory as the more expensive and less convenient warm chamber or microscope oven surrounding the microscope.

Illumination.—Light from the north and from a white cloud is the most desirable—or, south of the equator, a southern light. Direct sunlight or excessively bright light is to be avoided if possible as a source of illumination. Such high light intensities, if unavoidable, should be reduced by white shades or curtains. In the tropics a piece of plate glass fitted into the lower part of a wire screen frame gives good lighting, keeps out dust and does not interfere greatly with the circulation of the air.

Proper illumination is probably the most important point in microscopical technique; unless the light is utilized to the best advantage, the best results cannot

be obtained. In examining fresh blood preparations or hanging drops the concave mirror should be used and the light almost shut off by the iris diaphragm so as to give a contour picture. In examining a stained blood or bacterial preparation, the Abbé condenser should be properly focused so as best to illuminate the stained film. In many instruments set-screws are provided which check the elevation of the Abbé condenser when the proper focus is reached. Inasmuch as the light from the condenser should come to a focus exactly level with the object studied, in order to gain "critical illumination," it is evident that a fixed position for the condenser would not answer when slides of different thickness were used. It is best to use a slide about 1.5 mm. thick. Using daylight as described, or a special microscopists' lamp, the plane mirror should be employed when a color image is desired as in examining stained bacterial or blood films. If ordinary artificial light is used the concave mirror gives better results as such rays are not parallel. But by using a 6-inch globe filled with a very dilute solution of copper sulphate and a few drops of ammonia the rays are rendered practically parallel, and the light transmitted made to approximate daylight in quality, in which case the plane mirror should be used. Ordinarily in examining tissue sections, the Abbé condenser should be put out of focus either by racking down or by the use of the concave mirror and the narrowing of the aperture of the iris diaphragm. Very rarely one may wish to examine the cells of a section with the oil-immersion objective, in which case we use the condenser racked up fully and with the plane mirror instead of the concave one. It is only with the plane mirror that the rays are focused on the object by the condenser. Swing-out condensers are now made which are very convenient. The proper employment of illumination comes only with experience, and one should continue to manipulate his mirrors, diaphragm, and condenser until the best result is obtained. Then study the specimen.

An objection to artificial light is that one working almost entirely with daylight forms color standards and when using a different light is somewhat confused in interpretation of the microscopical picture.

There are now on the market special electric lamps with filters which give optical effects similar to those obtained with daylight. The bulbs of these lamps are filled with nitrogen gas, and the filter-screens have a bluish color. Considering the difficulties of securing daylight it would seem advisable to select artificial light as a standard illuminant.

Dark-ground Illumination.—Very valuable information, especially as regards the detection of treponemata in material from hard chancres or mucous patches, or the spirochaetes of relapsing fever, may be obtained by the use of dark-ground illumination. There are many different types of apparatus for this purpose.

The microörganisms under examination show as brilliant silvery objects against a dark background.

When the morphological details of a brightly illuminated object in the dark field can be distinctly observed, as with spirochaetes, it is proper to use the term dark-

ground illumination. When only particles, usually surrounded by bright and dark rings, and not showing any structure, are observed in the dark field the proper designation is ultramicroscopic. Ultramicroscopy deals with objects below 0.2μ which are too small for the resolving power of the microscope. In using the $\frac{1}{12}$-inch objective with dark-ground illumination a funnel-like base is supplied on which we screw the nickel-plated front mount of the objective. This funnel-shaped reducing diaphragm has to be inserted in the lower end of the foot of the objective to cut off rays which would interfere with the dark field. It is a method of reducing the N.A. to 1, or less. Before using the dark-field apparatus, which is screwed into the place of the Abbé condenser, it must be centered with a low power. This is carried out by getting concentric rings on the upper surface of the dark-field condenser parallel with the circle of the microscopic field. When using the immersion objective the ring or rings on the condenser extend beyond the circle of the microscopic field and one centers on a spot of white ink placed in the center of the ring on the condenser and deposited there while using a low power objective. The white ink spot is removed with moist lens paper before setting up the slide on the stage and making oil contact between its lower surface and the upper surface of the condenser. The centering is effected by centering screws in the condenser mounting. Immersion contact between the front surface of the condenser and the under surface of the slide carrying the preparation must be made before focusing the $\frac{1}{12}$th objective. As a source of illumination we may use a small arc lamp or a Nernst lamp or an incandescent gas lamp. The rays of light from these artificial sources should be made parallel by means of a water-bottle condenser and these rays projected on the plane mirror of the microscope. In using an arc-lamp one must have a suitable rheostat according to the electrical current employed. Information as to voltage and nature of current must be given the one supplying the apparatus. It is good practice to reserve a microscope for dark-ground illumination, so that it is always available for ready use.

In making preparations the slides and cover slips should be scrupulously clean and the material thinly spread out and free of bubbles. The slides should range in thickness from 1.45 to 1.55 mm., as thicker or thinner slides fail to allow proper focusing of the illuminator on the object. With low power objectives one can obtain satisfactory dark-field illumination by pasting a circle of black paper in the center of one of the glass discs which fit in the ring under the lens of the substage condenser. The diameter of the opaque center will have to be greater as the magnifying power of the objective increases and for oil-immersion objectives a special apparatus is required.

Apparatus for Sterilization

For the purpose of sterilizing glassware, media, and old cultures there are three methods ordinarily employed, the hot-air sterilizer, the Arnold sterilizer and the autoclave.

Since the nutritive properties of certain albuminous fluids used for the cultivation of bacteria are impaired by heat, it is customary to sterilize such media,

when necessary, by passing them, diluted, through a Berkefeld or other diatomaceous earth filter. Such filters hold back bacteria and give a sterile filtrate. In practice, however, ascitic fluid or serum is drawn and preserved with aseptic precautions and hence needs no sterilization.

Hot-air Sterilizer.—The hot-air sterilizer is ordinarily used for the sterilization of Petri plates, test tubes, pipettes, etc. For this purpose a temperature of about 150°C. is maintained for one hour.

If the temperature is allowed to go too high, there is danger of charring the cotton plugs of test tubes and also, due to the development of an empyreumatic oil, of making the plugs unsightly and causing them to stick to the glass. Further, we must be careful not to open the door until the temperature has fallen to 60°C.; otherwise there is danger of cracking the glassware. Where gas is not obtainable, the hot-air sterilizer is not a very satisfactory apparatus.

Arnold Sterilizer.—The Arnold sterilizer is to be found everywhere and can be used on blue-flame kerosene-oil stoves as readily as with gas burners. The most convenient form, but more expensive, is the Boston Board of Health pattern. The ordinary round pattern, with a telescoping outer portion, answers all purposes, however.

In the Arnold, sterilization is effected by streaming steam at 100°C. It is usual to maintain this temperature for fifteen to twenty-five minutes each day for three successive days. The success of this procedure—*fractional sterilization*—is due to the fact that many spores which were not killed at the first steaming have developed into vegetative forms within twenty-four hours, and when the steam is then applied such forms are destroyed. Experience has shown that all the spores have developed by the time of the third steaming, so that with this final application of heat we secure perfect sterilization. It is customary to use the Arnold for sterilizing gelatin, carbohydrate and milk media, even when the autoclave is at hand, the idea being that the greater heat of the autoclave may interfere with the quality of such media.

The Autoclave.—The most convenient autoclave is the horizontal type, such as is to be found everywhere for the sterilization of surgical dressings. The vertical autoclaves so much used formerly are awkward to handle and require more care.

The source of heat may be either electricity, gas, the Primus kerosene-oil lamp or steam from an adjacent boiler. By using Durham tubes—which are to be preferred except for gas analysis—even sugar media can be thus sterilized provided a very slight steam pressure be used. It is practicable also to inspissate blood serum tubes at a temperature of 103°C. in the autoclave. However, it is impossible to sterilize ordinary fermentation tubes in the autoclave on account of the boiling up of the media and the wetting of the plugs, and, if the ordinary tubes are used, they must be sterilized in the Arnold.

FIG. 3.—Bacteriological desk equipment. 1. Petri plates in holder. 2. Dissecting microscope. 3. Daylite lamp for dark field. 4. Disinfecting solution for used slides. 5. Wooden tooth picks. 6. Novy and Stewart forceps. 7. Jar for clean slides. 8. Bunsen burner. 9. Salt solution flask, salt cellar and bulb pipette. 10. Slide carrier. 11. Rectangular wire basket. 12. Tubes and rack for macroscopic agglutinations. 13. Magnifying tripod. 14. Glass tumbler with cotton in bottom for test tubes. 15. Water bottle in rack. 16. Substitute for small sink (see insert). Either Mohr or Hoffmann clamp may be used. 17. Bent glass rod container (alcohol in bottom). 18. Dropping bottles for stains. 19. Microscope. 20. Drawer for small apparatus, cover glasses, lens paper, etc.

Should a small bubble remain in the top of the small inverted inner tube after removal from the autoclave, one may make a mark with a grease pencil at the line of the bubble; or, if preferred, the basket of Durham tubes can be heated to boiling for ten minutes in a pan of water or in the Arnold and, after cooling, the bubble will be found to have disappeared.

Sterilizing glassware.—Glassware will come out from such an autoclave with wrappers as dry and plugs of the test tubes as stopper-like as could be effected in a hot-air sterilizer.

In sterilizing test tubes we place them in small rectangular wire baskets 6 × 5 × 4 inches. These baskets are to be preferred to round ones, as they pack more satisfactorily in the refrigerator used for storing media. In sterilizing flasks, test tubes, Petri dishes, throat swabs, pipettes, etc., it has been our custom, after exposing to 20 pounds' pressure for twenty minutes, to produce a vacuum for two or three minutes, and then to leave the steam in the outer jacket for a few minutes to thoroughly dry the articles in the disinfecting chamber. The valve to the inner chamber is then opened to break the vacuum; the door is now opened, and the articles removed in as dry a state as if they had been in the hot-air sterilizer. Articles, however, can be thoroughly dried in the sterilizer without the use of a vacuum by simply allowing the steam to remain in the outer jacket with the steam cut off from the inner chamber.

Pressure and Temperature Table

5 pounds pressure,	107.7°C.,	227°F.
10 pounds pressure,	115.5°C.,	240°F.
15 pounds pressure,	121.6°C.,	250°F.
20 pounds pressure,	126.6°C.,	260°F.
25 pounds pressure,	130.5°C.	267°F.
30 pounds pressure,	134.4°C.,	274°F.

Tests conducted at the U. S. Naval Medical School showed that in order to obtain and maintain the above pressure-temperature relations the sterilizer chamber must be free and kept free of a maximum amount of air during period in which steam enters chamber. This can be accomplished by keeping the chamber drain valve slightly but continuously open. Failure to properly expel air results in marked deviation from the normal pressure-temperature relation. For example: In one test where the air was not expelled until the chamber pressure reached 20 pounds the temperature within the chamber at 10 pounds pressure was only 78°F. and at 20 pounds pressure only 149°F.

All such articles **Petri dishes, pipettes, swabs,** etc., are wrapped in a cheap grade of filter paper, a fold being made and the ends turned in as is done in a druggist's package. Old newspapers answer well for this purpose.

Cleaning Glassware

It is a routine in our laboratory for everything to go through the sterilizer at 125°C. before anything else is done. This is a safe rule

when dealing with dangerous pathogenic organisms (especially tetanus and anthrax).

As soon as the tubes or dishes are taken out of the sterilizer they are emptied of their contents and placed in a 1% solution of washing soda and boiled. This thoroughly cleans them. As the washing soda slightly raises the boiling-point of the solution and also makes the spores more penetrable, it would be sufficient to place all contaminated articles in a dishpan with the soda solution, and boil for at least one hour, omitting the preliminary sterilization in the autoclave. The tubes are next cleaned with a test-tube brush, thoroughly rinsed with tap water and placed

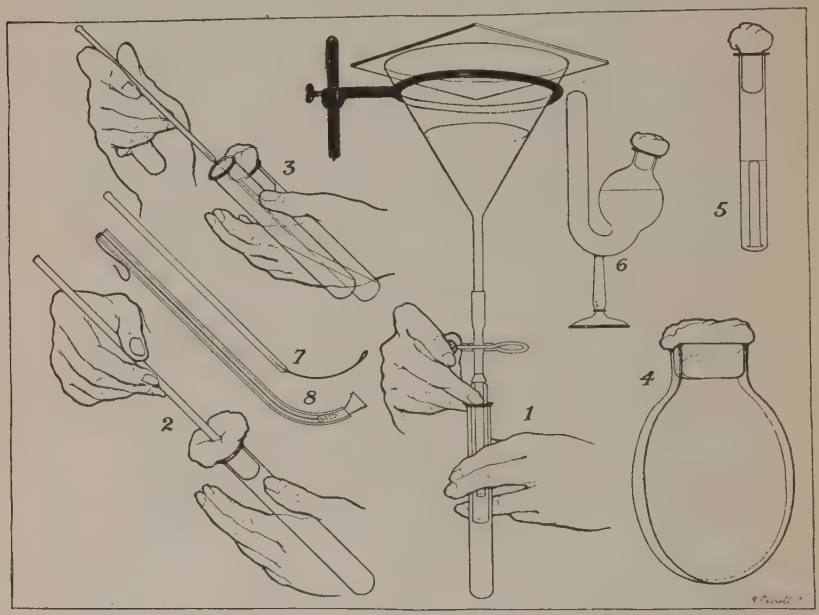


FIG. 4.—1, Filling tubes. 2, 3, Plugging tubes. 4, Culture flask plate. 5, Durham fermentation tube. 6, Smith fermentation tube. 7, Pharyngeal loop for meningococcus pharynx cultures. 8, West tube for same.

in a 1% solution of hydrochloric acid for a few minutes; then rinsed thoroughly in water and placed in test-tube baskets, mouth downward, and allowed to drain over night. Some laboratory workers boil their test tubes and other glassware in water containing soap or soap powder and, after a thorough rinsing in tap water, drain. Hydrochloric acid should not be used after the soap as it will cause the formation of an unsightly coating difficult to remove. When thoroughly dry, tubes may be plugged and sterilized.

Cleaning Fluid.—A cleaning fluid commonly used in laboratories consists of 1 part each of potassium bichromate and commercial sulphuric acid with 10 parts of

water. This is an excellent mixture for cleaning old slides, etc., especially when grease or balsam is to be removed. It is very corrosive, however.

In cleaning glassware for such tests as the colloidal gold it is essential that we use such a cleaning fluid. An efficient and less corrosive method for cleansing slides and cover glasses is to leave them over night in an acetic acid-alcohol mixture (two parts of glacial acetic acid to 100 parts of alcohol). After removing slides and cover glasses from this mixture, they are dried and polished; in addition, it is well to pass them through the flame of a Bunsen burner or alcohol lamp to remove every vestige of grease. The cover glasses should be exposed only momentarily to the flame to avoid warping. Ordinarily, rubbing between the thumb and forefinger with soap and water, then drying with an old piece of linen, and finally flaming will yield a perfect surface for making a bacterial preparation.

An excellent cleansing disinfectant is Liquor cresolis comp., U.S.P., which has a phenol coefficient of 1.87 to 3. A 5% solution is useful as a desk-jar disinfectant and in treating faeces, sputum, etc.

Concave Slides, Fermentation Tubes

The concave slide is ordinarily used for making hanging-drop preparations for the examinations of bacteria as to motility, presence of capsules, size and arrangement.

To prepare a hanging-drop preparation for the study of motility it is best to place a loopful of the young bouillon culture, or a loopful of salt solution into which is then emulsified a small amount of growth from an agar slant, in the center of the cover glass; now having applied with a brush a ring of vaseline around the concave depression in the slide we apply the slide as a cover to the cover glass which latter adheres to the ring of vaseline. The completed hanging-drop preparation can now be turned over and placed on the stage of the microscope.

An alternative method, equally good, consists in spreading a ring or square of vaseline—smaller than the cover glass to be used—in the middle of a plain slide. Then putting a loopful of salt solution in the center of the space, and inoculating with the culture to be studied, we finally cover it with a cover glass, gently pressing the margins down on the vaseline. This gives a preparation for the study of motility or agglutination which does not dry out for hours, and is easier to focus upon than the concave slide hanging-drop preparation.

Hanging Drop.—In examining a hanging drop first use a low-power objective and, having brought into focus the margin of the drop as a center line, change to a $\frac{1}{6}$- or $\frac{1}{8}$-inch objective.

By this procedure a thin layer of fluid is brought under the high dry objective instead of the deeper layer in the center of the drop. It is not advisable to use an immersion objective with a hanging-drop preparation owing to great difficulty in focusing and the possible detachment of the cover glass by sticking to the oil objective.

The light should be cut down to a minimum with the iris diaphragm and the concave mirror used. When we have finished examining the preparation the cover glass should be pushed over with the forceps so that a corner projects; then seizing the free portion with the forceps, lift up the cover glass and drop it into the disinfecting solution along with the slide.

FIG. 5.—Hanging drop, over hollow ground slide. (*Mac Neal.*)

Fermentation Tubes.—The fermentation tube with a bulb and closed arm is expensive, difficult to clean, and is easily broken. It is, however, convenient in the determination of the gas formula of an organism. As a substitute in the study of gas production and in water bacteriology, the Durham tube is to be recommended.

The Durham Tube.—Into a test tube, about 25×175 mm., we introduce the special sugar media, then drop down a small test tube (12×75 mm.) with its open

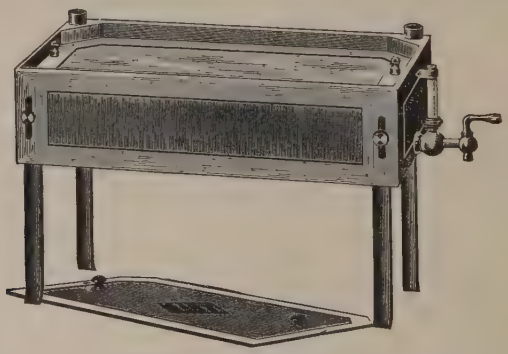


FIG. 6.—Blood serum coagulating apparatus.

end downward. Insert the plug of the large tube and sterilize. During sterilization the fluid enters the mouth of the smaller tube and fills it, and when the medium is subsequently inoculated, if gas forms, it appears in the upper part of the closed end of the smaller tube.

Inspissators.—For inspissating blood-serum slants a regular inspissator is desirable, this being nothing more than a double-walled vessel with the space between the walls filled with water.

As a substitute one may take the common rice cooker (double boiler). Fill the outer part with water; and in the inner compartment pack the serum tubes properly slanted on a piece of wood or wedge-shaped layer of cotton. Place a weight on the cover of the inner compartment to sink it into the surrounding water. Boil for one

or two hours. This same apparatus may be used for their sterilization, the process being repeated on two subsequent days, but it is better to sterilize in the autoclave or Arnold.

The rice cooker.—The rice cooker is of the greatest use in preparing culture media. For this purpose the outer compartment is filled with a 25% solution of calcium chloride or common salt, so that the temperature of the contents of the inner receptacle may be raised to the boiling point. Of course media may be prepared in an ordinary sauce-pan but there is great danger of scorching media prepared in this way. Special vessels with two bottoms, between which is an air space, are now on the market and have some advantage over the double boiler (rice cooker).

FIG. 7.—Rice cooker.

Laboratory Desk.—As regards a working desk, it will be found convenient to have an arrangement similar to the ordinary flat-top desk, with a tier of drawers on each side. A block of wood with holes bored in it to contain dropping-bottles may be placed in the upper right-hand drawer. In this way the stains are as accessible as if they encumbered the desk. It is advisable to paint the inside of this drawer black so as to reduce the deterioration which follows the exposure of many staining reagents to strong light. See Fig. 3.

Ebony Finish.—A very popular method of preparing the wooden surfaces of laboratory desks, sinks, and tables is the application of the so-called "acid-proofing." This gives an ebony-like finish which is not affected by strong acids. In using it, the surface of the wood must be new, or freshly planed, so as to be free of varnish, oil or paint.

Solution 1

Ferrous sulphate	20 Gm.
Copper sulphate	20 Gm.
Potassium permanganate	40 Gm.
Water, sufficient to make	500 cc.

Apply two coats of this solution with an interval of at least twelve hours between applications. When thoroughly dry apply two coats of solution No. 2.

Solution 2

Aniline oil	60 cc.
Hydrochloric acid	90 cc.
Water, sufficient to make	500 cc.

When the treated surface is thoroughly dry apply one coat of raw linseed oil with a cloth. After this is dry wash with very hot soapsuds. The excess of the black comes off during the first few days following application.

Useful Hints.—An aspirator bottle on a shelf elevated 2 feet, with rubber tubing and glass tip leading to a small aquarium jar or other desk receptacle, makes a good substitute for a small sink and faucet. A Hoffmann screw-clamp on the rubber tube controls the flow of water. The glass tube passes through a wooden clothes pin clamped on the edge of the jar. See Fig. 3.

Ordinary glass salt cellars will be found very useful as substitutes for laboratory watch glasses, or, after sterilization, to hold fluids for inoculating, etc.

A glass-topped fruit jar or a specimen jar containing a disinfecting solution for contaminated slides, etc., should be on every working desk. A good solution is that of Harrington (corrosive sublimate, 0.8; commercial HCl, 60.0 cc.; alcohol, 640.0 cc.; water, to 1000.0 cc.).

Graduated nursing bottles are very convenient for stock agar. We put about 150 cc. of medium in each bottle. These bottles are not fragile and withstand sterilization well.

There are many excellent types of water bath incubators on the market for use in Wassermann work. As a substitute in field work one may take an ordinary oval wash boiler and suspend in it, by wire supports bent over its edge, a heavy wire gauze diaphragm. Test-tube racks containing the Wassermann tubes are supported on this diaphragm. The boiler is filled with water up to the desired height above the supporting diaphragm. Once the temperature of the water is brought up to 37.5°C. the temperature can be maintained by a small flame under the wash boiler. For details see Fig. 10.

Plugging Test Tubes.—To plug a test tube, pick out a little pledget of plain absorbent cotton about 2 inches in diameter from a roll. Place it over the center of the tube and with a glass rod push the cotton down the tube about an inch. In culturing slow-growing organisms, as tubercle bacilli, or certain pathogenic protozoa, it is necessary to have plugs so prepared as to prevent drying out of the medium in the tube. The simplest way of accomplishing this is to melt some paraffin in a pan, then removing the cotton plug with the fingers to dip the end entering the tube into the melted paraffin and then push the surface so prepared into the tube. Plasticine, sealing wax or paraffin may be used to seal over the tops of such test tubes. We have found that impregnation of the cotton plugs with vaseline answers as well in keeping cultures from drying out as paraffin.

Sterile Swabs.—The sterile swab can be used for many purposes in the laboratory. It is most easily made by taking a piece of copper wire about 8 inches long, flattening one end with a stroke of a hammer, then twisting a small pledget of plain absorbent cotton around the flattened end. After wrapping, the swabs are sterilized in bunches. We not only use them for getting throat cultures, but in addition for culturing faeces, pus, or other such material. The material obtained with such a swab is then distributed in a tube of sterile bouillon or water. With the same swab the surface of an agar plate is stroked in a series of lines. This method is almost as satisfactory as the German one of using bent glass rods for this purpose. Everyone has encountered the difficulties attendant upon the bending of platinum wires and also the possibility of destroying organisms by an insufficiently cooled wire.

Platinum Needles and Loops.—For use in making loops and needles, platinum wire of 26 gauge will be found most suitable. Handles made of glass rod are preferable to those of metal. One end is fused in the flame and, holding the 3- to 4-in. piece of platinum wire, with forceps, in the same flame, insert the glowing metal into

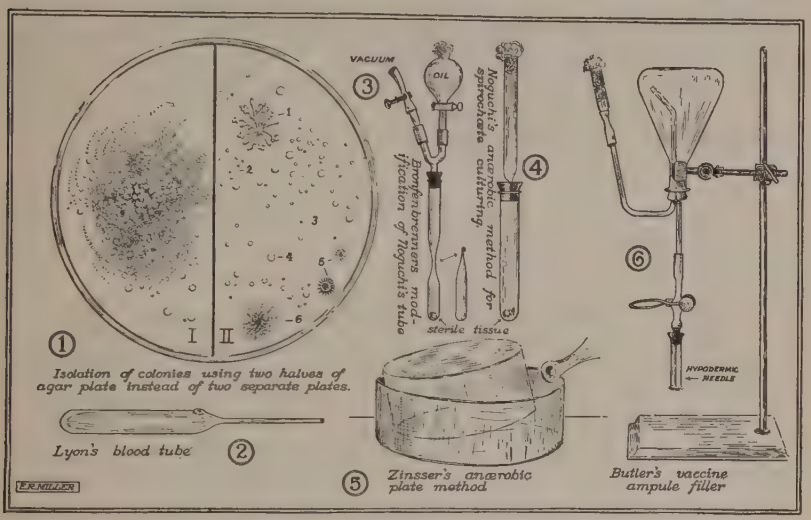


FIG. 8.—1, Method of using one plate instead of two for isolation of colonies (see page 71). The separated colonies on the No. II. side of the plate are studied with unaided eye and achromatic triplet ($\frac{1}{2}$ in. or $\frac{2}{3}$ in.) both by reflected and transmitted light. After we have determined the presence of two or more different kinds of colonies, a well isolated one of each type is selected and a blue pencil ring made around it on the glass surface of the back of the Petri dish together with a number (see Fig. 14). This number is carried along on culture tubes or microscopical slide preparations until the organism is identified. 2, Lyon tube for blood. More convenient than the Wright tube. For description see page 25. 3, Bronfenbrenner's anaerobic tube. This is made by drawing out a single test tube instead of using two separate tubes as with the Noguchi method. Before drawing out the test tube the piece of sterile tissue is introduced and after drawing out in the flame the ascitic broth or sheep serum water, as well as the material for culturing, is introduced through the drawn-out neck with a bulb capillary pipette. The lower part of the tube is placed in water at 37°C. and the vacuum connection made; after exhaustion of air the sterile paraffin oil is run in. The drawn-out portion is then sealed off in a small flame and looks like a sealed vaccine ampule. 4, Noguchi apparatus. 5, Zinsser method for anaerobic plates (page 114). 6, Butler's apparatus for filling vaccine ampules. The sterile vaccine is put in the sterile flask, and the stopper, with air intake and needle filler separately sterilized, then introduced into neck of flask which is then inverted.

the molten glass. By taking two lengths of platinum wire and twisting them together a more rigid needle is made for inoculating stab cultures. Loops and needles made from nichrome wire are as good as platinum ones and are very much cheaper. In making them, we saw a slit in aluminum rod handles and mash in with a vise the nichrome wire.

A platinum loop made around a piece of copper wire, 4 mm. in diameter, holds about 2 mg. of culture taken up from an agar slant. Kolle estimates that an agar slant of typhoid bacilli or of staphylococci should contain 15 such loopfuls while a streptococcus slant would have less than five. It has been estimated that such a standard loop would hold between 2,000,000,000 and 3,000,000,000 organisms. Of the greatest use in culturing material obtained at autopsy is the platinum spud. This can be made by hammering out one end of a piece of 15- to 18-gauge platinum wire. In smearing out material on the surface of a plate one may use a platinum or nichrome loop but these are apt to cut the surface of the medium. Sterile applicators, with a very little cotton covering and moistened in sterile salt solution, answer also, but the best "spreader" is a bent glass rod.

For making smears from faeces, sputum, and the like, wooden toothpicks are very convenient. The kind with the spatulate end is preferable.

Incubators.—When gas is obtainable, the maintaining of a constant temperature for the body temperature incubator (38°C.) and the paraffin oven (60°C.) is best secured by the use of some of the various types of thermo-regulators. The Reichert type is the one in general use, although there are many features about the Dunham and Roux regulators which are advantageous.

If the pressure of the gas supply varies from time to time, it is essential to regulate this by the use of a gas-pressure regulator (Murrill's is a cheap and satisfactory one).

Incubators, controlled electrically, are quoted in catalogues of American dealers. For our large incubator room we have used an electric control made by the Hixon Electric Co. which gave satisfaction. Such a room should be provided with an automatic recording thermometer. It is probable that the Koch petroleum lamp incubator is the most satisfactory one where gas is not obtainable. It should be of all-metal construction, and not have a wood casing, on account of the danger from fire. It costs from twenty-five to fifty dollars.

An incubator may be extemporized by putting the bulb of an incandescent electric lamp in a vessel of water. The proper temperature may be obtained by increasing the amount of water or by covering the opening more or less completely with a towel. The test tubes to be incubated can be put into a fruit jar or tin can, which receptacle is placed in the vessel heated by the lamp.

In emergencies we have used a chicken incubator for meningococcus plate work.

Emery suggests the use of a Thermos bottle as an incubator. The vacuum bottle should be first warmed by pouring in warm water. Afterward the bottle should be three-fourths filled with water at 100°F.

Schrup suspends his cultures and thermometer in the water by threads attached to pins in the cork of the vacuum bottle. The plug should be paraffined or covered with a rubber cap. There are now on the market large size vacuum containers for keeping food warm. These may be used in transferring plate cultures from a distance to the laboratory incubator.

Room temperature incubators.—As regards the need of a low temperature incubator for gelatin work, this may be met by using a small refrigerator. The ice in

the upper part maintains an even cold, and by connecting up an electric lamp in the lower part of the refrigerator we can easily maintain a temperature which varies only one or two degrees during the twenty-four hours. The gelatin plates or tubes should be placed on the shelves usually provided with the refrigerator and not on the bottom.

With a 16-candle-power lamp a temperature of about 25°C. is maintained (this is too high, being about the melting point of gelatin); with an 8-candle-power, one about 21° to 23°C.; and with a 4-candle-power, from 18° to 20°C.; the box being about 20 × 30 × 36 inches.

We have used with entire satisfaction a low temperature incubator made by Hearson.

The low temperature is produced by water from cracked ice packed in a large central chamber. A small dynamo controlled by a thermostat circulates the water around the chamber containing the gelatin cultures. It requires some time for

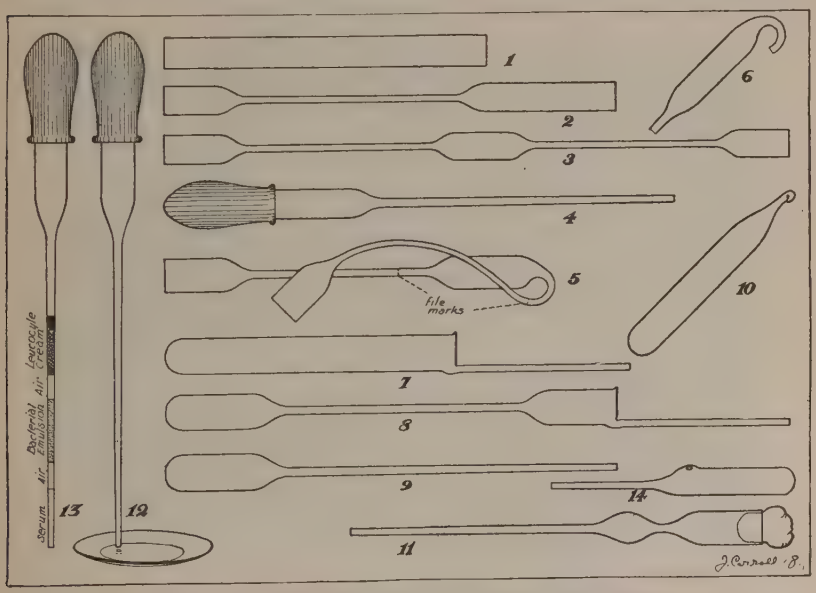


FIG. 9.—1, 2, 3, 5, 6, Wright's tube. 4, Rubber bulb pipette. 7, 8, 9, 10, Drawing out test tubes for stock vaccine. 11, Bulb bacteriological pipette. 12, 13, Pipettes for opsonic index work. 14, Lyon's blood tube.

proper adjustment, but afterward maintains a uniform temperature. Should the temperature of the room in which the incubator is installed fall below 22°C. there is provided an automatically controlled heating coil which then begins to operate.

Ice box incubator.—Certain laboratory procedures, for example the Kolmer Wassermann technique, call for an incubation at a temperature of 6° to 8°C. It is difficult to obtain and maintain a temperature below 10°C. with an ordinary ice box,

and certain electrical refrigerators, while satisfactory in operation, are expensive. Armstrong of the U.S.P.H.S. Hygienic Laboratory has devised a simple, inexpensive ice chest for this purpose. It consists of an upper compartment for ice, and a

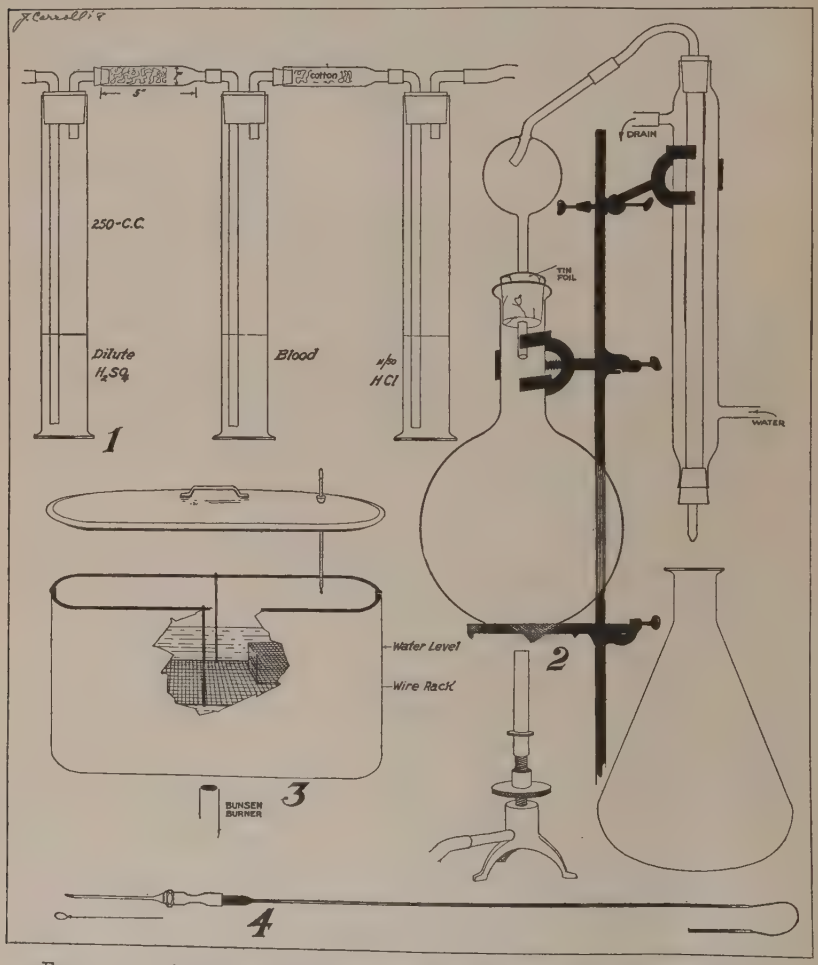


FIG. 10.—1. Apparatus to be connected with filter pump for blood urea determination. 2. Apparatus for distillation in making colloidal gold solution. 3. Substitute for expensive Wassermann water bath. 4. Capillary tube attached to needle for taking blood from vein in coagulation test.

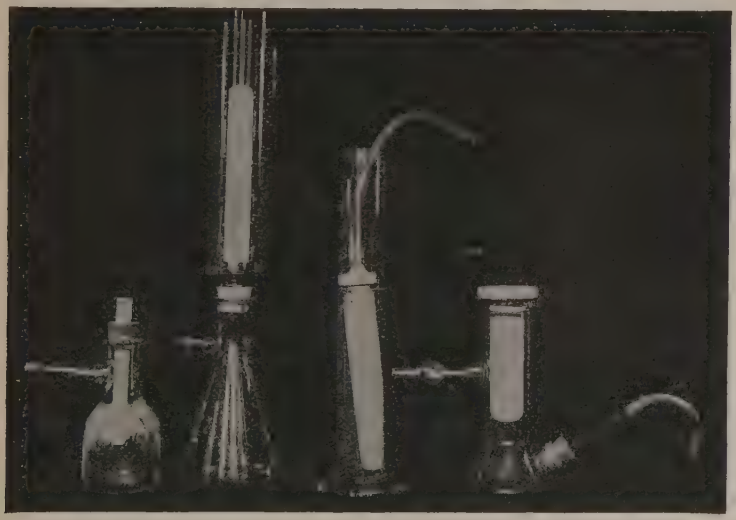
lower, larger compartment for refrigeration. The lower compartment is surrounded by a narrow air space continuous with the ice compartment. This permits water from the melting ice to drain down all sides, thus giving a maximum degree of

refrigeration. The external construction and insulation are those of the ordinary ice box.

Centrifuge.—When much serum reaction work is done, an electrically run centrifuge is an absolute necessity. It should be strongly constructed and placed on a firm base. There should be places for 8 tubes and the outer shell or guard should be so strong that in event of the breaking of a tube, while the centrifuge is revolving at high speed, there would be no danger for the operator. Water-power-driven centrifuges are less satisfactory and hand ones least so. The gyroscopic centrifuge is very satisfactory.

Drying Oven.—An electric drying oven is very useful, being in fact almost essential in determinations like that for blood acidosis where the steam vapor from the water bath interferes with the reaction.

Filter Pump.—A filter pump attached to the water faucet, preferably by screw threads, is almost indispensable for filtering cultures, etc., and for cleaning small



a *b* *c* *d*

FIG. 11.—Different types of bacteriologic filters; *a*, Kitasato; *b*, Berkefeld; *c*, Chamberland; *d*, Reichel.

pipettes, especially the haemacytometer pipettes. Such a filter or vacuum pump is more easily controlled when fitted with a vacuum gauge. In washing red cells in Wassermann reactions a pipette attached to the rubber tubing of the pump facilitates the removal of the supernatant fluid.

The filter pump is indispensable when using the various types of porcelain or Berkefeld filters. The Punkal or Muencke types of filter are the most convenient

in filtering toxins or in the sterilization of certain media when heating would be unadvisable. We have found the type "c" of the illustration the most satisfactory for general work.

Capillary Pipettes.—With the possible exception of the platinum loop, there is no piece of apparatus so generally useful as the capillary pipette made from a piece of glass tubing.

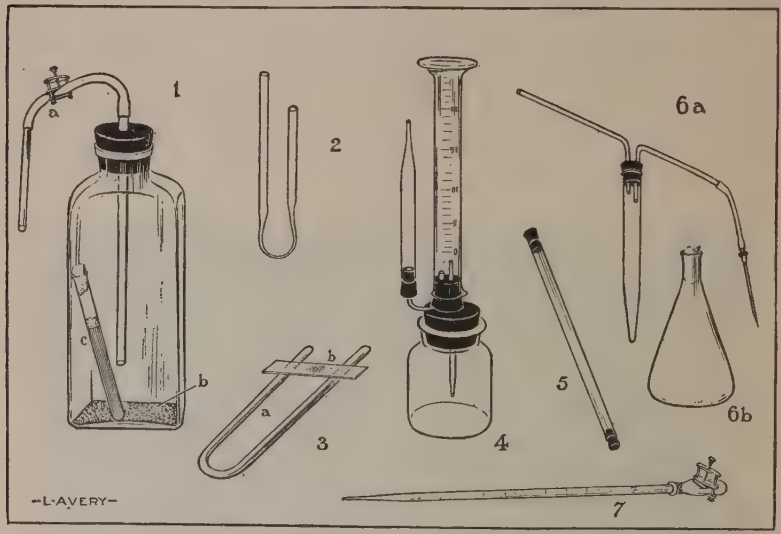


FIG. 12.—1, Apparatus combining various methods for culture of anaerobes; (*a*) Hoffmann clamp for connecting with vacuum pump; (*b*) pyrogallic acid at bottom of bottle for Buchner's O_2 absorption method; (*c*) deep glucose agar stab covered with sterile liquid petrolatum (see Anaerobes). 2, One-fourth inch capillary loop U-tube for making two nitric acid albumin tests (see Chemical examination of Urine). 3, Piece of tubing bent to hold slide for steaming smears in flame. 4, Schmidt's fermentation apparatus, as modified by using graduated cylinder (see under Faeces). 5, One-fourth-inch glass tubing, 4½-inches long with corks at each end. For centrifuging faeces for ova. 6*a*, Apparatus connected with sterile centrifuge tube for taking blood from vein of man or a guinea pig or rabbit's heart. 6*b*, Erlenmeyer flask which can be used instead of centrifuge tube. See under sections Immunity and Blood. 7, A graduated pipette with Hoffmann clamp applied to rubber bulb for precise delivery of measured quantities of liquids.

These may be made in a great variety of shapes. The Wright tube, with a hooked end which permits hanging the crook on the centrifuge guard, is the best known apparatus for securing blood for serum tests. By filing and breaking the thicker part of the tube, the serum is made directly accessible to a capillary rubber bulb pipette, or to the tip of a haemacytometer pipette, thereby facilitating dilution of the serum. See Fig. 9.

Lyon's blood tube.—Quite recently I have been using this blood tube. To make it, heat a 5- or 6-inch section of ½-inch tubing in the center and draw out as for making 2 bacteriological pipettes. Divide, and seal off the large end in the flame. Next seal off the capillary end. Then apply a very small flame to a point on the large end just before it begins to taper to the capillary part. The heat causes the heated sealed-off air inside to force out a blow hole. To use, break off the sealed capillary end and allow it to suck up blood from a drop just as with the Wright tube. I consider this tube superior to that of Wright.

The *capillary pipette* is made from a piece of ¼-inch soft glass tubing, about 6 inches long.

Held by the ends and constantly revolved, this is heated in a Bunsen flame, preferably fitted with a fish-tail tip, until it becomes soft in the center. It is then

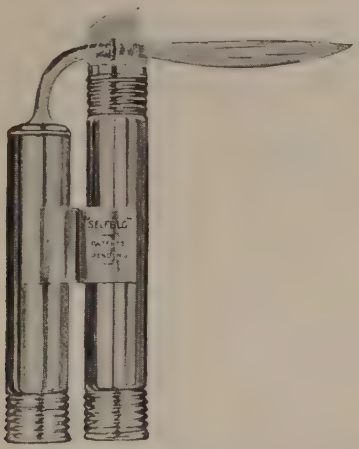


Fig. 13.—"Selfblo" automatic alcohol torch.

removed from the flame and, with steady traction, drawn out so that there intervenes a capillary portion 18 to 20 inches long. When cool, file and break off this capillary portion in the middle. We then have two capillary pipettes. By using a rubber bulb, such as comes on medicine droppers or larger ones, we have a means of sucking up and forcing out fluids. The bulb should be pushed on about ½- to ¾-inch; this gives a firmer surface to control the pressure on the bulb.

A *bacteriological pipette* (Fig. 9, insert 11), is made from a piece of tubing 9 inches long. Heat successively in the flame points 3 inches from each end, in each instance drawing out the tube just sufficiently to make a constriction. Then, following the procedure described in the preceding paragraph, there are obtained 2 pipettes similar to the one illustrated. A piece of cotton is lightly pushed into the large end.

Although these pipettes may be sterilized during the flaming, and used immediately afterward, it is better to keep on hand a supply, suitably wrapped and autoclaved for use on occasion.

Gas Substitutes.—Where gas is not at hand, the Barthel alcohol lamp gives a flame similar to that of the Bunsen lamp and is equally satisfactory for heating glass tubing. By making a collar with a lateral opening to fit the burner of a Primus lamp a powerful side-flame is obtained which is almost as suitable for glass blowing as the Bunsen blast usually employed. The ordinary plumbers' blast lamp also can be used.

One cannot appreciate the importance of a gas supply for a laboratory until he has experienced the lack of it. At present electricity is generally available but it cannot replace gas.

In the Philippines we had a Tirrill gasolene gas generator for 50 burners. The apparatus costs only about $500 and gives absolute satisfaction. It includes a tank for the gasolene, placed about 40 feet from the building, and a mixer and air pump which are placed in the cellar of the laboratory.

The Matthews gas machine, which weighs complete 1600 pounds, is quoted at $326.00 for a 50 burner plant.

CHAPTER II

CULTURE MEDIA

Most pathogenic bacteria require for their metabolism complex food-stuffs similar in composition and reaction to the fluids of the animal body. In general, a suitable medium for their cultivation contains moisture, salt, protein (amino acids) and a definite concentration of ionizable hydrogen. Infusions or watery extracts of meat furnish the basis of most culture media. Certain bacteria require enriching substances, such as hemoglobin, blood serum or carbohydrates for artificial cultivation outside the body.

The consistency of media is modified by the addition of suitable amounts of gelatin, agar or albumin; thus giving a semisolid or solid medium, depending on the amount of the various ingredients.

While there are certain advantages in sterilizing glass test tubes prior to filling them with media, yet this may be dispensed with—the sterilization after the media has been tubed being sufficient. If a dressing sterilizer is at hand, this is preferable for sterilizing such media as bouillon, potato, and agar, 10 to 15 pounds' pressure for fifteen minutes being employed. Milk should be sterilized with the Arnold, subjecting it to three steamings for twenty minutes on three successive days. Gelatin may be sterilized in either way, but preferably in the autoclave at 7 pounds' pressure for fifteen minutes. As soon as taken out of the sterilizer it should be cooled as quickly as possible in cold water. This procedure tends to prevent the lowering of the melting-point of the finished gelatin and also preserves its spissitude.

In making media a rice cooker, or the apparatus described below, is almost essential; at any rate, it is so if ease, expedition, and unfailing success in preparation are to be achieved. As it is necessary to make the contents of the inner compartment boil, the boiling point of the water in the outer compartment must be raised. This is done by using a 25% solution of common salt or a 20% solution of calcium chloride in the outer compartment instead of plain water. Should $CaCl_2$ be carried over to media in the inner compartment (as by thermometer) coagulation of albumin and clearing of media will be prevented.

A 15% solution of salt raises the boiling point 2.5°C.; a 20%, 3.5°C., and a 25%, 4.5°C. Calcium chloride in solutions of similar strength raises the boiling point to about the same degree.

Makers of apparatus for the bacteriological laboratory now furnish a vessel for making media which has two bottoms with an intervening air space. This hot-

air layer prevents the scorching of the media which is so liable to occur when a plain sauce-pan is used. Its advantages over the rice cooker are that time is saved in bringing the media to a boil, and also in the maintaining of a brisk boiling temperature.

Reaction of Media

The method of adjusting the reaction of media by means of titrable acidity is being gradually superseded by that based upon acidity due to ions,—hydrogen-ion concentration.

It has long been recognized that there is an optimum reaction of media for each organism, and certain of them require great exactness in this respect. It has been particularly in cultivating this latter class that the defects of the older method have become evident, they having been largely responsible for our frequent failures to secure suitable growths. The inadequacy of the older method is due to several factors. The titration end-point with phenolphthalein and bacteriological media is not easy to recognize, and it varies in practice among different laboratories. A certain tint once secured means a definite pH, but it is the usual practice to secure the final, desired reaction by the addition of acid after neutralization or by the use of less alkali than is necessary to neutralize. Results can be consistent only as long as the medium is prepared from *exactly* the same materials and by precisely the same technique. The consequence of variability is that this final adjustment practically results in a very indeterminate pH, and the final media are by no means universally comparable. Titration while hot greatly increases the variability. We are now confronted with the fact that our earlier data in this respect, determined by methods for titrable acidity, were based upon unsound premises and are of but little real value. I will, however, give the older method for such as still employ it, describing it as used with nutrient bouillon.

The expression "per cent. acid" (or "alkaline") indicates the reaction of media and its degree expressed as the number of cc. of N/1 acid (or alkali) which, added to 100 cc. of the media at the neutral point, would produce a reaction in kind and degree equal to that existing. Acidity is also represented by a plus sign preceding, and alkalinity by a minus sign.

Thus a 0.5% alkaline medium is one the alkalinity of which corresponds to that produced by the addition of 0.5 cc. of N/1 NaOH to 100 cc. of neutral medium and is represented by −0.5. Note that here, as elsewhere, perfect accuracy requires that the amount of bouillon taken should be the volume desired minus the amount of acid, or alkali, to be added.

Hydrogen-ion concentration.—See p. 687 for a brief discussion of this subject. It is now generally recognized that this is the preferable method for adjustment of reaction of media, and that its employment tends to accuracy of interpretation and reproducibility of conditions.

The Barnett-Chapman method (see p. 692) for determination of pH, although not the most accurate, is very generally serviceable for the adjustment of the reaction

of media. Place 10 drops of the indicator in a test tube, add 1 cc. medium, dilute to 5 cc. with distilled H_2O, and mix. Titrate with N/20 NaOH until the color matches that of the desired pH in the standard series. 50 times the amount of N/20 NaOH used will be the amount of N/1 NaOH to add per liter of medium to produce the desired pH. If, in making the titration, the volume is decidedly increased by the added NaOH, it is best to add H_2O to the same volume in the standard tubes before making the final comparison. Intrinsic color in the media despite dilution necessitates "backing" (see p. 693).

Sterilization practically always changes the pH of media. Heating in poor glassware accounts for a small part of the effect, but most is due to factors in the media, and the total change is practically always an increased acidity. Sugar media show the greatest alteration. The greatest effect will occur after a previously alkaline pH (7.8–9.0), less with acid media (5.0–6.2), and that with neutral media (6.6–7.4) is practically negligible. The usual change is less than 0.2, and the maximum to be anticipated is about 0.4. Agar media are most conveniently adjusted in the broth stage, the later addition of agar causing no appreciable change. A rough adjustment of pH can be obtained by using the old titration method in connection with different indicators. The medium is titrated in the cold to a certain end point, but no acid or alkali is then to be added after neutralization in the vain expectation of securing a certain pH. One simply adjusts the whole batch as indicated by the titration of the sample. The first faint greenish-blue with brom-thymol blue would mean a pH of about 6.2; the first faint pink with phenol red, cresol red, or phenolphthalein would mean about 7.0, 7.4, and 8.4 respectively. Other end points can be devised from a study of the list of indicators on page 689. The reaction of the newer media described on the succeeding pages is given according to the pH method. A table showing the optimum hydrogen-ion concentration for the cultivation of various bacteria is given on page 76.

Nutrient Bouillon

This may be made from either fresh beef or veal or from meat extract. Media from fresh meat are usually lighter in color and possibly clearer. In the Philippines, however, certain measures employed for the preservation of the meat made it very difficult to prepare clear bouillon from it, so that meat extract was used entirely. There is very little difference, if any, in the nutritive power of media made in either way.

Meat Infusion Bouillon

Round steak, or veal, free from fat, chopped fine.....	500 grams
Tap water.....	1000 cc.

Mix and let stand in ice-box for 24 hours. Heat in streaming steam for 1 hour. Squeeze through cheese cloth until 1000 cc. is obtained. Filter through paper and make up loss with water. Add 1 per cent. peptone and 0.5 per cent. sodium chloride, C.P. Heat in streaming steam for 30 minutes, determine reaction, and correct

to pH 7.6. Filter through paper and place in test tubes, about 10 cc. to each tube, and sterilize for 2 hours in streaming steam, or in autoclave 15 minutes at 15 pounds pressure.

Bouillon Made from Liebig's Meat Extract

Place in a mortar 3 grams of Liebig's extract, 10 grams of peptone and 5 grams of sodium chloride. Dissolve the white of one or two eggs in 1000 cc. of water. Then add this egg-white water, little by little, to the extract, peptone, and salt in the mortar until a brownish solution is obtained. Pour this into the inner compartment of a rice cooker; apply heat to the outer compartment containing the salt or calcium chloride solution, allow to come to a boil and to continue boiling for fifteen to twenty minutes. Do not stir. Place inner compartment on the scales, and its counterpoise and a one-kilo weight on the other side. Add water until the two arms balance. Filter and sterilize.

The reaction of media made with Liebig's meat extract rarely exceeds +0.75 (from +0.6 to +0.9). Consequently for growing bacteria it is unnecessary to titrate and adjust reaction unless unusual precision is required.

Giblet Broth

Liver, spleen, etc., may be used for making broth. The technique is the same as for beef or other meat, substituting 500 Gms. of the solid organs for the beef or other meat. Very often these broths exhibit a slight cloudiness, but this cannot be avoided.

The chief objections to fresh meat as a base are: 1. It takes more time and trouble. 2. The reaction, due to sarcolactic acid and acid salts, is quite acid, so that it is necessary to titrate and neutralize the excess of acidity. 3. The reaction of the finished media tends to change unless the boiling at the time of making was very prolonged. 4. It is not infrequent to have a heavy precipitate of phosphates thrown down at the time of sterilization, thus making it necessary to repeat the process of filtration and sterilization.

Titration of Media

Titration (hot).—Of the rather cloudy solution obtained, take up 10 cc. with a pipette and let it run out into a porcelain dish. Add 40 cc. of distilled or rain water and about six drops of a 0.5% phenolphthalein solution. (Phenolphthalein, 0.5 Gm.; dilute alcohol, 100 cc.) Bring the contents of the porcelain dish to a boil and continue boiling for one or two minutes in order to expel all CO_2. Now from a burette filled with decinormal sodium hydrate solution run in this solution until we have in the boiling diluted bouillon the development of a faint but distinct pink which is not dissipated on further boiling. It is more satisfactory to take burner from beneath the porcelain dish just before running in the N/10 solution, again boiling so soon as a pink color is obtained. Having obtained the light pink coloration we read off the number of cc. or fractions of a cc. of N/10 sodium hydrate solution added to produce the color. This number gives the acidity of the bouillon in percentage of N/1 acid solution.

If we took 100 cc. of the medium and put it in a beaker and then ran in N/1 NaOH solution from a burette, it will be readily understood that if we had to add 3.5 cc. of N/1 NaOH to obtain the pink color, it would show that the acidity of the 100 cc. of medium being tested corresponded to 3.5 cc. of N/1 acid solution, and that its acidity was equal to 3.5% of N/1 acid solution, or that its reaction was +3.5.

As N/1 NaOH solution is too corrosive for general use in a burette, and as 10 cc. of medium is more convenient to work with than 100 cc., we use a solution one-tenth the strength of the N/1 NaOH and we take only one-tenth of the 100 cc. of medium. In this way it is the same from a standpoint of directly reading off our percentage reaction as if we had 100 cc. of medium and used N/1 NaOH solution. The A. P. H. Association recommends 5 cc. of the medium and the use of N/20 NaOH; but as the N/10 NaOH is always at hand for titrating gastric juice, it is more commonly used.

Titration (*cold*).—The recommendations of the A. P. H. Association call for making the titration with the medium boiling, but should it be found difficult to carry on the titration while boiling, the end reaction may be fairly accurately determined in the cold. Deliver into a beaker from a pipette 10 cc. of the bouillon and make up to 50 cc. with distilled water and add 5 drops of 0.5% phenolphthalein solution. Then run in N/10 NaOH from a burette and continue to add the N/10 NaOH solution from the burette, drop by drop, until further addition fails to show any intensifying of the purplish-violet color at the spot where it came in contact with the diluted bouillon in the beaker. This marks the end reaction and corresponds to the faint pink of the end reaction obtained at the boiling point. A delicate pink obtained in the cold indicates about +0.7.

Having determined the percentage acidity of the 10 cc. sample tested, we easily calculate the number of cc. of N/1 NaOH solution required to be added to the 1000 cc. of bouillon to obtain a reaction corresponding to the neutral point of phenolphthalein. It is more exact to take the average of two titrations.

As 100 cc. of medium would require 3.5 cc., 1000 cc. would require 10 times as much, or 35 cc. N/1 NaOH solution. Having measured out and added 35 cc of the N/1 NaOH solution to the meat infusion, containing salt and peptone, we have a solution which is exactly neutral to phenolphthalein, or 0. It is usually considered that a reaction of about 1% acid is the optimum reaction for bacterial growth. Hence we should now add 1% of N/1 HCl solution to the medium. This would be accomplished by adding 10 cc. of N/1 HCl solution to the 1000 cc. of neutralized medium, and we would have a medium with a reaction of +1. If we desired a reaction of 1% alkalinity we would add an additional cc. of N/1 NaOH solution to every 100 cc. of the medium at 0, or 10 cc. for the 1000 cc. of medium. The reaction would then be −1.

As a matter of convenience, the usual method followed is to determine the reaction of the medium and, it always being more or less acid, to add only enough N/1 NaOH to reduce the acidity to the percentage desired, instead of neutralizing all the acidity present and then, in a second operation, restoring the acidity to the point desired.

Thus finding the acidity of the medium to be 3.5% and desiring to give it an acidity of 1%, we would add only 2.5 cc. of N/1 NaOH to every 100 cc. of medium or 25 cc. for the 1000 cc. of medium. The reaction would then be found to be +1.

The neutral point of litmus is not a sharp one, but it corresponds rather closely with a reaction of +1.5 to phenolphthalein.

Thymol blue as indicator.—McIntosh and Smart have proposed the use of thymolphthalein (thymol blue) as an indicator with titration carried on at room temperature. The first development of a dirty greenish-blue color seems to correspond with the pink of the phenolphthalein at boiling, and is a more easily obtained end reaction, the checking of results being more constant. Another end point, obtained by adding about 1 cc. more of N/1 NaOH per litre, is represented by the appearance of a blue color that does not intensify upon the further addition of alkali. Measure 10 cc. of the broth into a porcelain evaporating dish; add 25 cc. of pure distilled water. Have a control dish alongside prepared in exactly the same manner. Add 5 drops of a 0.5% alcoholic solution of thymolphthalein to the first dish. Then run in N/10 NaOH from a burette, stirring continuously, and looking for a darkening of color as compared with that of the control dish. Just before this point a precipitate of phosphates occurs. The point may be more aptly described as the disappearance of the yellow tint and the development of a bluish. Note the quantity of N/10 soda required to produce this change. In practice it is best to make four estimations, and the first in which a distinct blue has been produced is rejected. With the average data a calculation is made as to the amount of N/1 soda necessary to produce the same reaction in the rest of the broth. This amount of soda is then added and the flask shaken.

The alkaline broth is now brought to boiling to deposit the phosphates and then filtered free from them. The reaction is now adjusted as desired. The addition of 10 cc. N/1 HCl per liter of broth will give a reaction of approximately +1.

Clearing and filtration of media.—Albuminous material in culture media is coagulated at 65°C. and the fine particles in liquid media are removed by filtration through paper. Precipitates of phosphates in media may be removed by filtration after the media is cold, but these in no way interfere with bacterial growth. When eggs are used to clarify, the whole egg or dried egg albumen is used. One egg is used to each liter, shaken or well beaten with an equal amount of water and stirred into the cooled medium. With dried egg albumen use 10 Gms. dissolved in 20 cc. of water and add to one liter of medium. The temperature of the medium is gradually raised to boiling over the free flame or in the Arnold for about one hour.

Media which solidify on cooling are usually filtered through absorbent cotton in a glass funnel kept warm by a water jacket or in the Arnold sterilizer. A good grade long fiber absorbent cotton is essential so that the lengths of cotton may be split horizontally and the split portions placed crosswise and placed on a square of gauze or preferably coarse wire netting. This prevents the cotton from jamming the neck of the funnel and gives a broad filtering surface. The cotton is moistened with the medium and the first filtrates returned until it comes through clear. The coagulated albumins settle on the cotton and act as a part of the filter.

Filtration through paper pulp is carried out in the Buchner funnel using a suction pump. Filter paper is shredded and placed in a wide mouth bottle, using ¢ large

sheets of filter paper to 2½ liters of hot water. Shake vigorously at intervals for 48 hours until a fine uniform suspension is obtained. When ready, fit the bottom of the Buchner funnel with surgeons' lint, fleecy side uppermost. Dilute 400 to 500 cc. of pulp with 2 or 3 liters of hot water and pour carefully an even layer on the lint about one-fourth inch thick and cover with a sheet of filter paper. Place a suction flask under the funnel and apply the suction pump to draw the water into the flask until the paper is firm, but moist. Now add the hot agar slowly to prevent breaking the filter and discard the first 100 cc. which contains the water from the pulp. No egg is used when media is filtered by this method as it clogs the filter.

By sedimentation or by use of the Sharpless super-centrifuge all contact with paper or cotton is avoided in clearing and the "hormones" or "vitamins" are retained in the medium. See Huntoon's medium.

Nutrient Agar Agar

Take 1000 cc. of bouillon having a reaction of pH 7.6 and add a sufficient amount of N/1 NaOH to correct reaction to pH 8.0. Then add 15 grams of threaded agar, heat in streaming steam for 2 hours, or autoclave 30 minutes under 15 pounds pressure. Let stand in sterilizer overnight. Slip the cylinder of agar from the container, cut off the precipitate and discard it. Melt the clear portion and fill into test tubes, 5 cc. for slants and about 10 cc. for plates and stabs. Sterilize in streaming steam for 2 hours or autoclave 10 minutes at 15 pounds. The final reaction of this medium will be about pH 7.6.

Instead of threaded agar powdered agar may be used, but the following steps are required:

Sprinkle the powdered agar on the surface of the cool bouillon; let stand until the agar has sunk to the bottom; stir briskly; heat in streaming steam for 2 hours and cool the medium to 60°C. Constantly stirring, add the white of one egg beaten up with an equal amount of water, mix thoroughly, heat in streaming steam for one hour, then filter through a moist paper in a hot sterilizer.

Ordinary agar on the market is a variable product and there is no way of telling its solidifying quality unless each lot is tested for its *pectin* content, which should be present to not less than 90%.

Test for pectin.—Eight to ten grams of thread agar is washed rapidly in running cold water and dried overnight at 37° to 56°C. Then weigh out 5 Gms. and dissolve in 1000 cc. of distilled water containing 2% sodium chloride, place in Arnold or autoclave for one hour. When cool add 2000 cc. of 95% alcohol to precipitate the pectin. Let stand overnight, filter through weighed filter paper and dry the residue in incubator at 37°C. Reweigh the residue and paper, calculate the amount of residue and calculate the percentage of pectin.

Nutrient Gelatin

Take 1000 cc. of bouillon and 125 grams of "Best French Gold Label" or Nelson's photographic gelatin. Heat the bouillon and add the gelatin, stirring constantly. When the gelatin is in solution titrate and correct reaction to pH 7.6. Cool to 60°C. and add the white of one egg beaten up with an equal amount of water. Heat in streaming steam for 30 minutes, filter through paper and place in test tubes, about

10 cc. to each tube. Sterilize by the intermittent method, i.e., for 30 minutes in streaming steam on each of three successive days. Cool rapidly and store in a cool place.

Potato Medium

Cut with a cork borer or apple corer a cylindrical piece of potato about 6 cm. long. Cut this in halves diagonally lengthwise and place in cold running water for one hour. Place the potato pieces in large test tubes, each tube containing a wad of cotton saturated with water on the bottom. Sterilize in streaming steam for 2 hours.

Potato Agar Slants

Grind up 1000 Gms. white potatoes, add 1000 cc. water and cook until thick soupy liquid results. Strain through gauze and make up to 1000 cc. with distilled water. Add agar agar to make 3 per cent. Autoclave at 15 pounds pressure for 30 minutes. Filter through cotton and gauze. Tube in tubes 25 × 150. Autoclave for 15 minutes at 15 pounds pressure. Slant. This media may be used for all purposes for which glycerin potato slants are used by addition of 5% glycerin.

Sugar Bouillon

The sugar media ordinarily used for determining fermentation or gas production are those of glucose and lactose. In special work such carbohydrates as saccharose and maltose are used. The alcohol mannite is used in differentiating strains of dysentery bacilli.

These media are made by dissolving 1 or 2% of a sugar in sugar-free bouillon or that made from meat extract. Tube in Durham's or the ordinary fermentation tubes and sterilize in the autoclave at only about 5 pounds pressure for fifteen minutes, or in the Arnold. It is now considered essential that the sugar solutions, in 20% strength, be sterilized separately and then added to the sterile bouillon. 0.5 cc. of the 20% solution added to 10 cc. of medium would give a 1% solution.

Too high a degree of heat may turn the sugar bouillon brownish. The nature of the sugar itself may further be affected by too high a temperature, since many of the carbohydrates, when in bouillon, are liable to be split up on subjection to any marked sterilization. *Maltose* is particularly unstable. *Inulin* usually contains resistant spores so that its sterilization may need the autoclave. One of the great difficulties about reporting on sugar reactions is the possibility of not working with a chemically pure sugar as well as with one changed by too much heat. Inulin is a polysaccharid resembling starch but does not give the iodine reaction. It is obtained from the roots of chicory or dandelion. For further notes on carbohydrates see chapters on the study and identification of bacteria and on water analysis. We color our sugar bouillon as noted under indicators. When it is desired to determine percentage of acid or lowered pH value produced by an organism in carbohydrate media do not add indicator until making titration or comparing with standard pH tubes.

Sugar-free Bouillon

Round steak, free from fat, chopped fine	500 Gms.
Tap water	1000 cc.

Mix and let stand in a cool place for 24 hours. Strain through cheese cloth until 1000 cc. of fluid are obtained. Inoculate the meat juice with 10 cc. of a 24-hour bouillon culture of B. coli and place in incubator at 37°C. for 24 hours. Heat in streaming steam for 1 hour. Filter through paper and make up loss with water to original volume. Titrate and correct the reaction to pH 7.6. Add 1 per cent peptone. Heat in streaming steam for 30 minutes and again titrate and correct the reaction to pH 7.6. Filter through paper and sterilize for 2 hours in streaming steam.

For ordinary purposes the very small amount of sugar in bouillon made from Liebig's meat extract may be neglected in determining gas production; the various sugars may be added directly to the meat-extract bouillon. Dunham's peptone solution may be used as a substitute for sugar-free bouillon, particular sugars being added as desired. We usually employ the serum water medium of Hiss, but it must be remembered that serum contains glucose (blood sugar), so that for critical work this medium should not be used.

Enlow's Sugar-free Medium for Fermentation Studies

Tap or distilled water	1800.0 cc.
Peptone	10.0 Gms.
Na_2HPO_4	14.5 Gms.
KH_2PO_4	1.4 Gms.
Agar	2.0 Gms.

These substances are heated together in the Arnold sterilizer for 40 minutes, or autoclaved for 10 minutes at 15 pounds, filtered through paper while hot, and diluted with hot distilled water to 2000 cc. The salts added as indicated make adjustment unnecessary for most organisms. The pH will vary from 7.2 to 7.4. The sugars, alcohols, glucosides, etc., are added in 0.5% amounts, followed by the indicator, after which the medium is tubed and sterilized by the fractional method (30 minutes in Arnold sterilizer on 3 successive days). The bromthymol blue is added in amount sufficient to give a distinct green-blue or deep-blue color (depending upon the pH desired). If the indicator is prepared as given below, 3 drops in each 500 cc. is a sufficient amount to give a very positive color change due to increased hydrogen-ion concentration.

The indicator may be prepared as follows:

Bromthymol blue (dibromothymol sulphonphthalein)	0.1 Gm.
N/20 NaOH	3.2 cc.

Grind in agate mortar. When solution is complete add 3 cc. of distilled water.

The formula given above will produce a medium varying from pH 7.2 to 7.4, depending upon the nature of the ingredients used.

The salts and peptone tend to stabilize the medium but the buffer strength is not sufficient to prevent appreciable change in the hydrogen-ion concentration resulting from attack of the fermentable substance. If a higher or lower pH value is desired, the salts can be varied according to Sorenson's table.

As control on the growth of the organism in this medium without the addition of fermentable substances, it is necessary to prepare one lot of tubes without such

substances, that is, using merely the water, peptone, potassium and sodium salts, agar and indicator. In case attack is made upon the peptone which might be considered as obscuring the results of the carbohydrate attack, this will be indicated in these tubes, which we have termed "non-fermentable controls."

Cooked Meat Medium for Anaerobes

Chopped meat	1 part
Tap water	2 parts

Mix and heat in Arnold sterilizer from 1 to 1½ hours. Filter through filter paper and allow to stand for 1 hour. Distribute meat into large sized test tubes. Adjust reaction of broth to pH 8.5. Distribute broth into test tubes containing the meat, so that the broth and meat are about equal in volume. Sterilize 1½ hours at 15 pounds pressure. Add 1 to 2 cc. sterile vaseline. Heat 1½ hours in Arnold sterilizer. Final reaction is about pH 6.8 to 7.0.

Calcium Carbonate Bouillon

When we wish to cultivate such organisms as streptococci and pneumococci in massive cultures we may add small fragments of marble (calcium carbonate) so that any inimical excess of acid may be neutralized. Addition of a small piece of calf's brain and 0.5% dextrose as used by Rosenow is quite satisfactory.

Glycerin Bouillon

Add 6% of glycerin to ordinary bouillon. It is used chiefly in the cultivation of tubercle bacilli.

Peptone Solution (Dunham's)

Dissolve 1% of Witte's peptone and 0.5% of sodium chloride in distilled water. Filter, tube, and sterilize. Peptone solution may be used as a base for sugar media instead of bouillon.

Note.—If the peptone gives an extreme acidity, it may be necessary to titrate and correct the reaction to pH 7.6. This is particularly essential in growing the cholera vibrio.

Indol Test.—This test is made by adding from 6 to 8 drops of concentrated H_2SO_4 to a twenty-four to forty-eight-hour-old peptone solution culture of the organism to be tested. If the organism produces both indol and a nitroso body we obtain a violet-pink coloration, "cholera red." If no pink color is produced on the addition of the sulphuric acid, add about 1 cc. of an exceedingly dilute solution (1:10,000) of sodium nitrite.

Another satisfactory method is to add to a three-day growth in plain broth, 1 cc. of concentrated HCl (or any strong mineral acid). Mix thoroughly and overlay the acid broth with 1 or 2 cc. of a 0.1% solution of sodium nitrite. At the junction of the two solutions a brownish-red ring develops if indol is present.

The *Vanillin* test also gives satisfactory results. Five drops of 5% vanillin solution in 95% alcohol and 1 cc. of strong sulphuric acid are added to about 5 cc.

of the culture to be tested. The presence of indol is indicated by the formation of an orange color; the presence of tryptophan of a reddish-violet.

Cholera Red.—It is very important in determining the "cholera red" reaction to know that the peptone used will give the reaction as it is not given by true cholera strains with certain samples of peptone.

Voges-Proskauer (Acetyl-methyl-carbinol) Reaction.—For this test, fill fermentation tubes with a 2% glucose Dunham's peptone solution and sterilize. After inoculation with the organism to be tested incubate for three days. Then add 2 to 3 cc. of strong caustic potash solution. The development of a pink color on exposure to the air is a positive reaction, the color being that of a weak eosin solution.

Medium for Methyl Red Test

Distilled water	800 cc.
Difco or Witte's peptone	5 Gms.
Dextrose	5 Gms.
Dipotassium hydrogen phosphate, K_2HPO_4	5 Gms.

Heat with occasional stirring over steam for 20 minutes, filter through paper, cool to 20°C., and dilute to 1000 cc. with distilled water. Fill in test tubes, about 10 cc. to each tube, and sterilize by the intermittent method for 20 minutes on three successive days.

For preparation of the methyl red indicator solution and use of the test see page 223.

Holman's Serum Broth

Take 200 cc. of double strength nutrient bouillon (+1.2), add 100 cc. water, 4 grams of test carbohydrate and 4 cc. Andrade's indicator. Sterilize on three succesive days in the Arnold. Beef serum is diluted one-half with water and filtered through a Berkefeld filter and 200 cc. of this sterile diluted beef serum is added to the bouillon just described. The serum bouillon is then tubed and incubated to eliminate contamination.

Hiss' Serum Water Medium

Take one part of clear beef serum and add to it about three times its bulk of water. Heat the mixture in the Arnold for fifteen minutes to destroy any diastatic ferment which might be present. Color to a deep transparent blue with litmus solution and then add 1% of the sugar to be used. Sterilize in the Arnold by the fractional method.

Glucose Agar

Add the agar to 1 or 2% glucose bouillon and proceed as for ordinary agar. If preferred, the glucose agar can be made by rubbing up meat extract 3 grams, peptone 10 grams, salt 5 grams, glucose 10 grams and 15 grams of agar in 1000 cc. of water containing the white of one to two eggs, then boiling in the rice cooker and filtering.

Glycerin Agar

Add the sugar to 6% glycerin bouillon, instead of to nutrient bouillon, or the glycerin may be added to nutrient agar which has been melted. Glycerin agar with a reaction of o makes an excellent base for blood and serum media for use in culturing delicate pathogens.

Glycerin Agar Egg Medium

Take the white and the yolk of one egg and mix thoroughly with an equal amount of glycerin agar in a vessel kept between 45° and 55°C. Tube the medium, inspissate in a rice cooker as for serum tubes, and sterilize as for blood-serum tubes.

This makes an excellent medium for growing tubercle bacilli. As egg medium has a tendency to be dry, it is well to add 1 cc. of glycerin bouillon to each slant before autoclaving.

Agar Gelatin Medium (North)

Lean chopped beef or veal	500 grams.
Agar	10 grams.
Gelatin, Gold Label	20 grams.
Peptone, Witte's	20 grams.
Sodium chloride	5 grams.
Distilled water, *q.s.*	1000 cc.

Extract the chopped beef with 500 cc. distilled water for eighteen hours, strain through muslin and combine the ingredients in the usual way. Adjust the reaction to the neutral point, using phenolphthalein as indicator.

North states that this medium is excellent for streptococci, pneumococci and diphtheria bacilli because it is soft, moist, and can be used at 37°C.

It is claimed to be of special value for carrying stock cultures. It is useful as a plating medium for milk.

Litmus Milk

Milk for media should be as fresh as possible. As soon as received, it should be put in a 1000-cc. Erlenmeyer flask, sterilized for fifteen minutes in the Arnold, and set over night in the refrigerator. The next morning the milk beneath the cream should be siphoned off, care being taken that the short arm of the siphon does not reach the bottom of the flask so as to avoid including sediment. Add sufficient litmus solution to this milk to give a decided lilac tinge; tube and sterilize in the Arnold on three successive days.

Litmus milk which apparently is as satisfactory as the above as regards nutritive quality and cultural characteristics can be made from certain canned milks which have not been condensed or sweetened and which do not contain chemical preservatives. The "Natura" brand of milk is the one with which I have experimented.

Indicators

Litmus solution.—A simple solution may be made by digesting the powdered cubes repeatedly with hot water, mixing the extracts, and, after allowing them to stand all night, decanting the solution from the inert sediment into a clean bottle.

A solution so made, however, contains not only the essential blue dye but also a red dye together with soluble calcium and other salts, and, while answering the demands of routine work, is not adequate for bacteriological purposes. In this work, there should be used a solution of the blue dye in a pure form called "azolitmin."

It can be conveniently prepared as follows: Weigh out 2 ounces of powdered litmus; digest repeatedly with fresh quantities of hot water until all the coloring matter is dissolved out; allow to settle, and decant the fluid from the insoluble powder. Add together the extracts, which should measure about a liter. Evaporate down the solution to a moderate bulk, then add a slight excess of acetic acid, so as to convert all carbonates present into acetates. Continue the evaporation, the later stages over a water bath, until the solution becomes pasty. Add 200 cc. of alcohol, and mix thoroughly. The alcohol precipitates the blue coloring matter, while a red coloring matter, together with the alkaline acetate present, remains in solution. Transfer to a filter. Wash out the dish with alcohol and add this to the filter. Wash the precipitate on the filter with alcohol. Dissolve the pure coloring matter remaining on the filter in warm distilled water and dilute to 500 cc. Azolitmin solution prepared in this way is more sensitive than ordinary litmus solution.

Azolitmin in powder can be purchased from dealers in chemicals. It is used as a reagent in 5% aqueous solution neutralized with N/1 NaOH, 1 cc. of this solution being added to 100 cc. of liquid sugar media or of Russell's double sugar agar.

Neutral red.—We have recently been using neutral red to color certain liquid sugar media, adding 0.2 cc. of a 1% aqueous solution of this indicator to each 100 cc. of medium. In the presence of an acid, neutral red becomes a deep rose-red color, while an alkali turns it yellow.

Andrade's indicator.—This indicator has come into general use as a substitute for the litmus indicator as showing acid production in carbohydrate media. To prepare it, take of acid fuchsin 0.5 Gm. and distilled water 100 cc. To this magenta-red solution add N/1 NaOH until the color changes to pink, then to brownish-red and then to yellow. Shake the reagent after each addition of the alkali. Usually it takes about 17 cc. of normal sodium hydrate solution to decolorize 100 cc. of the fuchsin.

Add 1 cc. of the decolorized reagent to 100 cc. of any sugar bouillon. The broth is colorless at room or incubator temperatures, but pink at 100°C. If an organism produces acid it turns the indicator magenta red.

By confining the use of certain indicators to certain media it is possible to differentiate uninoculated tubes without the necessity of labeling them. Our practice is as follows: With large (150 mm.) tubes, neutral red for glucose, Andrade for lactose and litmus for saccharose; with small (100 mm.) tubes, neutral red for maltose, Andrade for mannite and litmus for inulin.

When inoculating the tubes and placing them in the rack it is of some importance to retain the sequence of media as listed above. The red, white and blue colors in the fresh tubes serve as a memory key. Further, the acid end reactions of neutral red and litmus are occasionally sufficiently alike to be confusing, whereas if separated by the bright color in the Andrade tube the possibility of confusion is lessened.

Newer indicators.—Bromcresol purple, phenol red and cresol red are the most useful indicators as these may be added directly to the medium in the minute

quantities necessary without being reduced by bacterial growth as are litmus and methyl red. The choice of the indicator depends on the initial reaction of the finished medium and as bacterial growth proceeds the changes in the pH values of the medium may be studied and recorded by comparing with a known standard from day to day. The amount of the indicator used in the medium is unimportant so long as a distinct color is obtained, as these indicators do not affect the bacterial growth. We have found that 1 cc. of a 1.6% alcoholic solution to a liter of the medium is usually satisfactory. When making comparisons with the standard pH tubes proportionate amounts of the indicator are used.

Blood Serum

The blood of cattle should be collected in large pans or pails at the abattoir. The vessel of blood should then be kept in the cold-storage room and the next morning the more or less clear serum will have been squeezed out from the clot. Collect this serum and keep in the ice chest for future use. If to be kept for a long time, it is advisable to add about 2% of chloroform to the serum in tightly corked flasks. This will not only keep the serum, but will eventually sterilize it.

To make *Löffler's serum*, take 1 part of glucose bouillon and 3 parts of blood serum. Mix, tube, and coagulate in the inspissator or rice cooker, giving the tubes a proper slant before heating. Sterilize on the following day in the autoclave as previously directed (7 pounds) or in the Arnold on three successive days. See directions on page 11.

Blood serum is preferably solidified as slants in a blood-serum inspissator. This requires one to two hours. The subsequent sterilization in the autoclave or Arnold should not be done immediately after making the solidified slants, but on the subsequent day. If done on the same day, many of the slants are ruined by being disrupted by bubbles.

The autoclave as an inspissator.—When large numbers of blood-serum tubes are to be inspissated it is more convenient to use an autoclave. A screen frame is made to fit the inner chamber of the sterilizer and set at the desired angle for proper slant. The tubes are put in and steam is turned into the outer chamber only, never permitting the heat to rise above 3 pounds or 105°C. During the process of inspissation, which usually requires from 45 to 60 minutes, the door of the sterilizer should be opened occasionally in order to keep the temperature below the point at which bubbles are generated in the media.

The rice cooker as an inspissator.—The preparation of blood-serum slants or slants of egg media can be conveniently carried out in a rice cooker (double boiler). Place the tubes in the inner compartment of the cooker, obtaining the slant desired by manipulating an empty test tube, or with a towel or cotton batting on the bottom. Then cover the tubes with another towel. The outer compartment should contain water alone (not 25% salt solution). The inner compartment should be weighted down so that it is surrounded by water—the weight of the light tubes not being sufficient to sink it. With the water in the outer compartment boiling, one or two hours will suffice to inspissate or solidify the slants satisfactorily. The sterilization on subsequent days is best done in an Arnold or an autoclave, since the rice cooker, if used for this purpose, makes the media too dry.

Greenspon's medium (for *B. diphtheriae*).—Preparation: To 75 cc. of clear pig, sheep or human serum is added 1 cc. of a 50% sodium citrate solution and sufficient dextrose veal infusion broth (see p. 29) to bring the volume to 100 cc. Beef extract broth may be substituted for the meat infusion. With 3% citric acid solution the reaction is then adjusted to pH 6.4, using dibromthymol-blue as an indicator. Next the medium is delivered into culture tubes, coagulated in an inspissator and sterilized by the fractional method on three successive days.

This medium is said to be superior to Löffler's serum in that, through the action of the citric acid, the growth of many of the common mouth organisms is inhibited and the growth of the diphtheria bacillus is accelerated.

A Substitute for Ordinary Blood Serum

Add from 10 to 15 cc. of 1% glucose bouillon to the white and yolk of one egg, make a smooth mixture in a mortar, and tube.

By flaming the mortar wet with alcohol, and by using a sterile knife for cracking the egg, it is possible so to reduce the bacterial content that sterilization of the medium can be accomplished as the result of the two-hour inspissation in the rice cooker. If these precautions are not taken, it will be necessary to sterilize after inspissation as for ordinary serum slants.

By covering the tube with a rubber cap or, preferably, by heating the plugged end of the test tube, quickly withdrawing the cotton plug and dipping the part of the plug which enters the tube into hot melted paraffin, then quickly reintroducing the plug, the contents of the tube will be prevented from drying out. This procedure is essential for growing tubercle bacilli. It is also well to protect such cultures from the light by wrapping opaque paper around the tube.

When the medium is to be used for culturing tubercle bacilli, about 1 cc. of glycerin bouillon should be added to each tube before final sterilization in the autoclave. While glycerin bouillon favors growth of human strains of tubercle bacilli, it is not so satisfactory for bovine strains as plain glucose bouillon.

This medium seems to answer as a substitute for Dorsett's egg medium and is superior to the various white-of-egg substitutes usually recommended.

In morphology and luxuriance of growth, cultures of diphtheria bacilli on this medium are similar to those on Löffler's.

Egg Media for Culturing Anaerobes

The medium described above, with the addition of 1% neutral red solution in proportion of 5 drops to each egg, is of value in anaerobic work in that it makes colonies producing acid more distinctly visible.

Dorsett's Egg Medium

This is prepared by breaking whole eggs into a sterile flask, mixing thoroughly, then adding 25 cc. water to every 4 eggs, straining through a sterile cloth and tubing 10-cc. quantities. These tubes are slanted in an inspissator and kept at 73°C. for four or five hours on two successive days. On the third day a temperature of 76°C. is applied. Before inoculating add 3 or 4 drops of sterile water to each tube. Tuberculosis material should be rubbed into the surface well and the plugs paraffined.

Lubenau's Egg Medium

Put 10 fresh eggs in a 5 per cent. solution of carbolic acid for 4 or 5 minutes. Wipe them dry with a sterile towel. Puncture both ends with a sharp pointed sterile instrument. Blow the contents of the egg through the larger end into a sterile flask. Add 200 cc. of a 5 per cent. glycerin bouillon previously sterilized.

Mix thoroughly by shaking, strain through sterile cheese cloth, and fill into sterile test tubes by means of a sterile filling funnel. Place the tubes in a slanting position in the Arnold sterilizer. Bring the temperature slowly up to 85°C. allowing 1½ hours to reach this temperature. Sterilize at 85°C. for 3 hours.

Petroff Medium

Round steak or veal, chopped fine..... 500 Gms.
15 per cent glycerin in water..... 500 cc.

Mix and let stand in a cool place for 24 hours. Strain through cheese cloth.

Mix 1 part of the glycerin meat juice and 2 parts of whole egg. To this add 1/10,000 part of gentian violet.

Mix and strain through cheese cloth, fill into tubes and place in Arnold sterilizer in a slanting position. Bring the temperature slowly up to 85°C. allowing 1½ hours to reach this temperature. On the second and third day heat to 75°C. for one hour each day.

Hydrocele, Serum, Ascitic and Milk Agar

To tubes of melted 2 or 3% agar at 50°C. add from 1 to 3 cc. of hydrocele or ascitic fluid, observing aseptic precautions. Allow the agar to solidify as a slant, or as a poured plate.

For milk agar we add 2 or 3 cc. of plain or litmus milk to a tube of melted agar. This makes an excellent plating medium for *B. bulgaricus*. When poured into a plate it is opaque but the colonies stand out well.

For obtaining sterile serum we generally use the apparatus described under Blood Agar and as a rule take the blood from vein of arm in man or jugular vein of neck of sheep. The sterile serum separates from the clot and can be pipetted off with a sterile pipette and added to the melted agar. A method recommended by Fildes is to collect blood at the slaughter house in sterile vessels, allow to clot and remove serum. Five cc. of ether is added to each 100 cc. in a bottle with a ground stopper. After fixing the stopper securely in place, the mixture is heated for one hour in a water bath at 45°C., after which it is placed in the incubator at 37°C. for several days, by which time it is sterile. Before using the serum the ether is driven off at 45°C. This is better than the old method of sterilizing with 2% chloroform.

Blood Agar

For obtaining blood to make blood agar we use the apparatus described under blood culturing and shown in Fig. 12. We take human blood from the arm vein,

sheep blood from the neck vein, or rabbit blood from the heart. In an Erlenmeyer flask of 100-cc. capacity is placed 5 cc. of sterile 5% sodium citrate solution, provided we want to take 50 cc. of blood from sheep or man. The final mixture, to prevent coagulation, should contain about 0.5% sodium citrate. As a rule we take only about 25 cc. from man or rabbit so 2.5 cc. of 5% citrate would usually suffice. The perforated rubber stopper is removed from the flask and replaced with a sterile cotton plug. When needed, the fluid mixture is pipetted off and added to melted agar.

Mixture of blood and agar is facilitated by rotating the tube rapidly between the hands. The medium may be slanted or poured into plates. As this medium is satisfactory for the growth of haemoglobinophilic organisms, as well as for others, we use it as a routine plating medium, together with nutrient agar and Endo.

Oleate haemoglobin agar.—To make Avery's oleate haemoglobin agar add 5 cc. of 2.2% solution of neutral sodium oleate to 95 cc. to nutrient agar which should have pH of from 7.2 to 7.5. Outside of this range of reaction the Pfeiffer bacillus does not grow. While the oleate agar is still hot add defibrinated or citrated blood. Oleate blood agar is probably the most favorable medium for culturing the Pfeiffer bacillus, but cooked blood agar and ordinary blood agar are very satisfactory.

Whole blood agar for differentiating streptococci (Brown).—The base is 500 Gm. veal, 5 Gm. salt, 15 Gm. agar and 10 Gm. peptone per liter of media, prepared as for ordinary nutrient agar. The final reaction is from +.8 to +1.2. The medium is tubed in 12-cc. amounts. To prepare blood agar melt down the base and place in water bath at 45°C. The tubes should be kept in the water bath about 15 minutes before adding 0.6 cc. of defibrinated blood and mixing thoroughly. The blood agar at 45°C. is now inoculated with a loop or two of 24-hour culture of the streptococcus to be studied and then poured into Petri dishes to give a depth of 2 mm. This medium is also satisfactory for meningococcus plating.

To defibrinate blood we take broken pieces of glass tubing or rod, drop them into an Erlenmeyer flask and sterilize. The blood is taken as with the citrate method. The standard technique is to use glass beads instead of fragments of tubes, but cast-off pieces of capillary tubing answer well.

Chocolate (cooked) blood agar.—Melt down nutrient agar or preferably glycerin agar and while the temperature of the medium in the tubes is about 90°C. add about 4 or 5% of citrated blood—0.5 cc. to 10 cc. agar in tube. Mix thoroughly, avoiding bubbles, and pour into plates. This cooked blood agar gives a luxuriant growth of Pfeiffer bacillus but is a dirty brownish opaque medium and is less satisfactory for isolating colonies than the ordinary blood agar.

Pure blood media.—For culturing the bacillus of chancroid (Ducrey) we use whole rabbit or human blood taken aseptically and preserved in small test tubes. The blood should be inactivated by heating the tubes for ½ hour at 56°C. This is a suitable medium for maintaining the virulence of pneumococci and streptococci.

In preparation of the agar base used for blood plates it has been found at the United States Naval Medical School that ordinary nutrient agar (2 to 3%) made up with Liebig's beef extract, salt, and peptone in the usual amounts gives satisfactory results. Many workers, however, insist on meat infusion agar; some laboratories have doubled the amount of peptone; some have omitted using salt, and some have added 1 to 2% glucose.

Huntoon's Hormone Agar

Beef heart, free from fat and finely chopped	500 Gms.
Whole egg	1
Peptone	10 Gms.
Sodium chloride	5 Gms.
Agar, pulverized	18 Gms.
Tap water	1000 cc.

Mix in an enamelware dish in a water bath and heat (constantly stirring) until the color changes to brown—68 to 70°C. Titrate by adding 4 per cent. NaOH until the medium reacts slightly alkaline to litmus paper. Add 1 cc. more of 4 per cent. NaOH. Cover the vessel and place in an Arnold sterilizer for 1½ hours, or water bath at 100°C. Remove and separate the firm clot from the sides and return to Arnold sterilizer for 1½ hours. Remove and tip gently, allowing the fluid portion to accumulate at one side, which is now removed by means of a large pipette or siphon, avoiding any fat floating on the surface. Allow the agar to stand in a cylinder for 15 minutes. Skim off any fat present. Tube and sterilize in the usual manner.

If further clearing is desired, accomplish by sedimentation, filtration through glass wool, or centrifugation. *The medium at no time must come in contact with cloth, cotton, or filter paper.*

Plates made from this medium should be dried in the incubator for 1 hour before inoculation.

Slant agar should be allowed to stand 24 hours before inoculation. For preserving stock cultures, the medium is the same as above, but only 5 grams of agar are used. Inoculate by stab method.

Fluid medium.—Same as above, but substitute for agar 1 to 2% gelatin.

Selective Media for Gonococci

During the last few years there has been a good deal written on the importance of reduced oxygen tension in culturing gonococci. Investigators have suggested, however, that it is not so much the reduced tension that has been responsible for the good growths obtained as the fact that the methods employed to maintain this tension have insured the presence of an increased amount of moisture.

Swartz's medium.—This probably is the best known medium in which reduced oxygen tension is employed. It is made from a beef or veal infusion agar, prepared in the ordinary manner and brought to a reaction of pH 7.6, phenolsulphonephthalein being used as an indicator in hot medium. When the medium has cooled to about 50°C., add the white of three fresh eggs to each 1000 cc. Starting with a low flame, boil for 10 minutes, strain through cloth and then filter through filter paper. Place 5 to 6 cc. of the medium in each test tube and autoclave at 10 pounds pressure on three successive days. This sterilization reduces the pH to 7.4. Now add sterile ascitic, pleuritic or hydrocele fluid to the melted agar in the proportion of one part of fluid to two parts of agar. The tubes are then sealed with sterile rubber stoppers and slanted. Corking, by preventing evaporation, permits storage of the media in

the incubator, this, in turn, facilitating the detection of contamination and keeping the medium warm for inoculation at any time. The use of the rubber stopper has a further advantage in that it prevents contamination much more surely than the ordinary cotton stopper.

The medium is to be inoculated as richly as possible. It is important also to avoid letting the material cool pending inoculation and to have the medium at body temperature when inoculated.

Immediately after inoculation, the tube, held horizontal, is turned so that the agar slant is uppermost. Held by the butt, it is then passed longitudinally through the Bunsen flame about three or four times and quickly corked. Experiments with suitable apparatus show that this procedure heats the air in the test tube sufficiently to reduce the pressure within, when the tube has become cool, from 70 to 100 mm. of Hg (about 10% of atmospheric pressure) and, when properly carried out, does not coagulate the medium nor impair the viability of the culture. When the agar has set, the tubes should have about 0.5 cc. of water of condensation in the lower angle of the slant. This, according to Swartz, assures the best growth. The period of viability of the gonococcus on this medium is about seven days.

Swartz in an original report presents the following conclusions:

1. The superior growth of gonococcus in closed systems, when part of the air or of the oxygen has been removed or replaced, is essentially due to the lowered oxygen tension, and not to moisture, change of reaction, or presence of CO_2.

2. Moisture is however necessary to good growth.

3. A reduction in the oxygen tension of 10 per cent. is sufficient to produce optimal growth.

4. The gonococcus will grow luxuriantly, if the oxygen tension is suitable and moisture and uncoagulated proteid are present, on media having an initial reaction anywhere between pH 6.6 or pH 8.0 inclusive.

5. In dextrose-containing media, the acid end point for the gonococcus is pH 5.6.

Pelouze and Vitcri's calf brain agar.—A new medium is presented that is as simple to make as ordinary agar. It can be autoclaved after completion; it gives as many positive first cultures; it grows the gonococcus indefinitely in subcultures and retains its viability for at least a month.

A calf's brain, weighing approximately 500 Gms. is forced through a wide-meshed gauze into 500 cc. of distilled water and placed in the ice-box for 24 hours. It is then filtered several times through cotton of varying degrees of compactness. The resultant fluid is turbid, no matter how often it is filtered. To this is added 0.5 per cent. acid sodium phosphate and 1 per cent. of peptone. It is then autoclaved at 15 pounds pressure for 20 minutes and kept as stock, or the final steps for its completion can be carried out.

To complete the medium it is necessary simply to add one part of the brain bouillon to three parts of standard 2.5 per cent. agar medium made from veal broth, with the addition of 0.5 per cent. of sodium chloride and one per cent. of peptone. It should then be adjusted to a pH of 7.8 to allow for change, 7.6 being desired endpoint.

The medium is then tubed, autoclaved and slanted. After it solidifies the usual cotton plug is replaced by a sterile rubber stopper to retain the water of condensation, and the medium keeps indefinitely.

When it is completed and cooled, some flocculation is present in the butt of the tube. This can be easily removed if the medium in bulk is placed in the autoclave, quickly brought to 15 pounds pressure, removed, filtered, tubed and replaced in the autoclave for the completion of sterilization. While this improves its appearance, it seems to make some change in it which causes more scanty growths, consequently we have not made use of it, preferring a good medium rather than a pretty one.

Serum glucose cystine agar medium (Francis).—This medium is used for cultivation of *Bacterium tularense* but will also give abundant growths of old cultures of gonococcus and diphtheria bacillus. It is made as follows: Beef infusion containing 1% peptone, 1 or 1.5% agar and 0.5% NaCl and adjusted to a pH of 7.3 is kept on hand in stock. When needed there is added to the stock agar 0.1% cystine or cystein hydrochloride and 1% of glucose. This is placed for 15 minutes at the temperature of flowing steam in an Arnold sterilizer, to melt the agar and sterilize the cystine. The medium is now cooled to 45–50°C., and 5% sterile horse serum is added. The medium is then tubed, slanted and incubated for 24 hours to insure sterility.

Blood-streaked Agar

Clean the lobe of the ear and puncture with a sterile needle. Collect the exuding blood on a large platinum loop and smear it over the surface of an agar slant. It is advisable to incubate over night as a test for sterility. In preparing this medium or culturing such organisms as pneumococci, streptococci, gonococci and meningococci, it is well to use glycerin agar, pH 7.6, as the base.

Bile Media

Secure ox bile from the abattoir or human bile from patients undergoing gall-bladder drainage in hospitals. Put about 10 cc. in each tube and sterilize. Some prefer to add 1% of peptone. Conradi's medium is ox bile containing 10% of glycerin and 2% of peptone. This is the medium commonly used for blood cultures in suspected typhoid.

The *bile lactose medium* used in water analysis is made by adding 1% of lactose to ox bile and tubing in fermentation tubes. As a substitute for fresh bile one may use a 15 to 20% solution of a good quality of inspissated ox gall (Fel Bovis Purificatum). A *liver bouillon* made by using 500 grams of finely divided beef liver in 1000 cc. of water with 1% peptone, and prepared as for meat infusion broth, is a good substitute for bile.

Plating Media for the Intestinal Bacteria

Of the numerous media proposed for plating out faeces, probably the most satisfactory are the Endo, the Teague and lactose litmus agar. In making any of these media, the following base is recommended in place of those advised in the original formulae. It is more easily prepared and yields equally good media.

Liebig's extract	5 grams.
Salt	5 grams.
Peptone	10 grams.
Agar	30 grams.
Water to make	1000 cc.

Prepare as for ordinary nutrient agar, with the difference that the reaction should be brought down to 0. Some prefer a reaction of +0.2.

A stiff agar (3%) is employed to check the diffusion of acid beyond the colony.

Endo medium.—Keep agar base in 100-cc. quantities in Erlenmeyer flasks instead of test tubes. If more convenient smaller quantities may be put in the flask. When needed for plating, melt a flask of this agar, and while liquid add to the 100 cc. 6 drops of a saturated alcoholic solution of basic fuchsin, and then about 20 drops of a freshly prepared 10% solution of sodium sulphite. The sulphite solution decolorizes the intense red of the fuchsin to a light rose pink. This color fades to a light flesh or pale salmon color when cold. Now add 5 cc. of a freshly prepared hot aqueous 20% solution of chemically pure lactose. If only occasionally using such media, tube in 20-cc. quantities, adding fuchsin, sulphite and lactose in proportionate amounts. This medium contains 1% of lactose. Kendall prepared an Endo medium which contains only 1.5% of agar and with a reaction just alkaline to litmus (about +1.2 to phenolphthalein).

Colon bacilli show on this medium as vermilion colonies, which in about thirty-six hours have a metallic scum on them. Typhoid and dysentery colonies are grayish; streptococci, a deep red.

The "Standard Methods" Endo medium.—This medium as modified in the 1925 edition of Standard Methods of Water Analysis consists of a 3% agar, containing 5 Gm. of beef extract and 10 Gm. of peptone per 1000 cc. and adjusted to pH 7.8–8.2. It is flasked, sterilized and stored in convenient quantities. When wanted to use, melt 100 cc. of agar in streaming steam and proceed as follows adding the ingredients in order given:

One per cent chemically pure lactose in sterile solution, 0.5 cc. of stock basic fuchsin solution (10% alcoholic solution) and 0.125 Gm. anhydrous sodium sulphite dissolved in a small amount of hot distilled water. The sulphite solution must be made up fresh each time. Mix thoroughly.

Pour plates at once and allow to harden thoroughly in the incubator before use.

Lactose litmus agar.—Color 100 cc. of neutral agar base to a lilac color with azolitmin. Then add 5 cc. of hot freshly prepared 20% lactose solution in distilled water. This may be tubed, putting 10 cc. in each test tube, or put in quantities of 50 or 100 cc. in small Erlenmeyer flasks. It is then sterilized in the autoclave at 10 pounds for fifteen minutes, or in the Arnold. The addition of brom-cresol-purple or phenol red (1 cc. of 1.6% solution to the liter) is now used in preference to litmus.

Conradi-Drigalski medium.—To 100 cc. of lactose litmus agar add 1 cc. of a 1 to 1000 solution of crystal violet in distilled water. The medium is then ready to put into plates. Colon colonies are pink; typhoid and dysentery colonies, bluish-gray.

Teague medium.—We have formerly preferred the Endo plate for typhoid work and the lactose litmus agar when culturing faeces for dysentery bacilli. More recently we have obtained most satisfactory results with the Teague medium. On this, colon colonies, after eighteen hours, are deep-black and opaque while those of the typhoid-dysentery group are colorless and transparent. After thirty-six hours the plates are not very satisfactory.

The medium is prepared as follows: Nutrient agar is made in the usual way, containing 1.5% agar, 1% Witte's peptone, 0.5% sodium chloride, and 0.5% Lie-

big's meat extract, to the liter of distilled water. It is cleared with egg-white, placed in flasks, and sterilized in the Arnold sterilizer on three successive days. The reaction is brought to +0.8. The agar is melted, and saccharose 0.5% and lactose 0.5% are added. The medium is then heated for ten minutes in the Arnold. To every 50 cc. of the medium are added 1 cc. of 2% yellowish eosin and 1 cc. of 0.5% methylene blue. The mixture is shaken and plates poured. Eosin solution should be added first.

Conradi's brilliant green medium.—Take of Liebig's extract 20 grams, peptone 10 grams, agar 30 grams and water to 1000 cc. This amount of meat extract should give about the proper acidity, +0.3. If not, the reaction should be adjusted to that point. Filter through cotton, place 150-cc. amounts in 250-cc. Erlenmeyer flasks and sterilize.

Then add 1 cc. of a 1 to 1000 aqueous solution of brilliant green and 1 cc. of a 1% solution of picric acid to the flasks each containing 150 cc. of the melted agar. Sterilization after adding the dyes precipitates them and is unnecessary. Pour the finished med um into large Petri dishes and inoculate the surface with the faeces.

Brilliant green does not interfere with agglutination as does malachite green.

This medium is considered by some authorities the one of choice in isolating typhoid bacilli from faeces and urine.

The surface of the poured plates of Endo, Conradi-Drigalski, and the brilliant green media should be dried in the incubator before smearing with the faeces. For routine work I prefer the Endo medium followed by Russell's double sugar agar.

Eosin methylene-blue agar.—Add 10 Gm. of Difco peptone, 2 Gm. of dipotassium phosphate (K_2HPO_4) and 15 Gm. of undried agar to 1000 cc. of distilled water. Boil until all ingredients are dissolved and make up any loss due to evaporation with distilled water. Adjustment of reaction is not necessary.

Place measured quantities (100 or 200 cc.) in flasks or bottles and sterilize in the autoclave as directed at 15 lbs. for 15 minutes.

Just prior to using, melt stock agar and add the following ingredients to each 100 cc.: Lactose, sterile 20% solution, 5 cc. Eosin, yellowish, 2% aqueous solution, 2 cc. Methylene-blue, 0.5% aqueous solution, 2 cc. Mix thoroughly, pour into Petri dishes, allow to harden and inoculate by streaking on the surface.

It is allowable to add all the ingredients to the stock agar at the time of preparation, place in tubes or flasks and sterilize. Decolorization of the medium occurs during sterilization. The color returns after cooling.

Russell's Double Sugar Agar

To a fairly stiff agar (2 to 3%), with a reaction of about +0.7, is added enough azolitmin solution to produce a distinct purple-violet color. It may be necessary to add more alkali. To this litmus-tinted agar is added 1% of lactose and 0.1% of glucose, and the medium as thus prepared is tubed and slanted. Sterilization should be carried on in the Arnold, on two successive days, as the autoclave temperatures tend to break up the sugars. Andrade's indicator is better than litmus.

To inoculate this medium we take material from a suspected colony grown on Endo and smear the material on the slant; then with the same platinum needle we stab into the butt.

On these slants typhoid shows a delicate growth on the violet slant with a deep pink in the butt of the tube. The paratyphoids show gas bubbles in a pink butt with a violet slant. The colon bacillus turns both slant and butt a deep pink, and the butt is filled with gas bubbles.

Lead Acetate Medium

To make this medium, take 1.5% nutrient agar and add 1% glucose, 1% lactose and 0.05% basic lead acetate. The basic lead acetate solution is made up as a 0.5% solution and sterilized and the requisite amount added to each tube before slanting. The same technique is followed with this medium as with Russell's, stabbing into the butt of the tube and streaking the slant.

This medium is useful in differentiating the paratyphoids. Paratyphoid B organisms give a brownish discoloration which is not given by typhoid or paratyphoid A. The gas production is similar to that with the Russell tube.

Selective Media for Cholera

Dieudonne's medium.—The value of Dieudonne's medium rests on the ability of cholera to grow when alkali is present in such amounts as to inhibit the growth of other faecal bacteria.

Take equal parts of defibrinated blood obtained at the slaughter house and normal NaOH solution. Mix 30 parts of this alkaline blood mixture with 70 parts of hot 3% nutrient agar. The poured plates should be left half open over night in the incubator; otherwise even cholera will not grow on the plates.

Krumwiede's medium.—Krumwiede's formula is as follows. Take equal parts of whole egg and water, and add to the mixture an equal volume of 12.5% crystal sodium carbonate solution. Having steamed this alkaline egg mixture for twenty minutes, add 30 parts to 70 parts of meat extract-free 3% agar. (No meat extract; only peptone and salt.) The cholera colony has a hazy look, like a little wad of absorbent cotton sticking to the surface, with a metallic luster halo.

Goldberger's medium.—First prepare a 100% meat infusion by treating 500 grams of finely chopped lean beef with 500 cc. water and after three hours strain the infusion, adjust reaction to neutral with 5.3% anhydrous sodium carbonate, then add to each 100 cc. 2.5 cc. of the 5.3% anhydrous sodium carbonate, sterilize in Arnold for one-half hour and filter. Next prepare a 3% meat extract agar and mix one volume of the alkaline meat infusion with 3 volumes of the hot melted 3% meat extract agar. Pour plates and cover with a piece of filter paper and place in incubator for one-half hour until they are quite dry. The necessity for a surface without moisture applies to Dieudonne's and Krumwiede's alkaline egg media as well as this one. On this medium cholera grows well while faecal bacteria are restrained.

The cholera colony is clear, round and shows a brownish center but is without that striking bluish opalescence shown on ordinary agar plates.

Esch medium.—This medium has been highly recommended. It is easy to make. Heat 500 grams chopped-up beef with 250 cc. normal NaOH solution in a pot and when disintegrated filter through cloth and sterilize. About 1 part of this alkaline extract is added to 2½ to 2 parts of nutrient agar. The plates must be dry. The transparency of this medium is an advantage.

Aronson's medium.—This is an excellent medium in the examination of stools of cholera carriers. The organisms taken from such plates emulsify easily and there is no interference with their agglutinability. To prepare it add to 100 cc. of 3% nutrient agar, 6 cc. of 10% solution of exsiccated sodium carbonate and steam in Arnold sterilizer for fifteen minutes. Then add 5 cc. of 20% saccharose solution, 5 cc. of 20% dextrin solution, 0.4 cc. saturated alcoholic basic fuchsin and 2 cc. of 10% sodium sulphite. A precipitate forms which quickly settles and plates can be poured from the supernatant fluid. Cholera colonies develop in twelve hours and show as red colonies in fifteen to twenty hours. Colon colonies are much larger than these and are colorless. In stock cultures of the cholera vibrio the colonies are much slower in development.

Other selective media for cholera are those of Kabeshima in which a haemoglobin extract is treated with alkalis and added to nutrient agar.

Culture Media for Intestinal Protozoa

Boeck and Drbohlav's media.—*E. histolytica* and intestinal flagellates have been successfully cultivated on the following media.

(1) *Locke egg-serum or L.E.S. meaium* which is prepared as follows.

Four eggs are washed, brushed with alcohol and broken into a sterile flask containing glass beads. Fifty cc. of Locke's physiological solution are added and the mixture broken up by shaking. Test tubes are then filled with a sufficient quantity to produce slants from about 1 to 1½ inches upon coagulation by heat. These tubes are now slanted in an inspissator and heated (70°C.) until the egg mixture has solidified. They are then transferred to the autoclave and sterilized for 20 minutes at 15 pounds pressure.

The tubes are now covered to a depth of 1 cm. above the egg slant with a mixture composed of 8 parts of sterile Locke's solution and one part of sterile inactivated human blood serum. They are then incubated to determine sterility.

Locke's Solution

Distilled water	1000.00 cc.
NaCl	9.0 Gms.
$CaCl_2$	0.2 Gms.
KCl	0.4 Gms.
$NaHCO_3$	0.2 Gms.
Glucose	2.5 Gms.

The solution is sterilized, either in the Arnold or in the autoclave, according to the ordinary methods. The human serum when not sterile may be diluted with 2 or 3 parts of Locke's solution and then passed through a sterilized Berkefeld filter (No. N) which removes the bacteria. Sometimes a second filtration may be necessary. Bouillon tubes and agar plates are inoculated and incubated to determine the sterility of the filtrate. If the filtrate is not sterile, it may be passed through another filter (sterile) and new sterility tests made. When the filtrate proves to be sterile it is further diluted with the necessary amount of Locke's solution to give the concentration of 8 parts of Locke's solution to 1 part of serum.

(2) *Locke egg-albumin or L.E.A. medium* which is prepared by covering the egg slants with Locke's solution containing 1% of crystallized egg albumin. It has the advantage over the L.E.S. medium of being more readily prepared since the albumin is usually more available than human serum.

The best growth of amoebae occurs between pH 7.2 and 7.8 which is usually the pH of the L.E.S. and L.E.A. media. Adjustment, however, may be required.

Craig recommends for the cultivation of *E. histolytica* the following media (1) Locke's solution 7 parts and inactivated horse, rabbit or human blood serum 1 part, (2) modified Ringer's solution 7 parts and inactivated human blood serum 1 part and (3) normal salt solution (0.85) 7 parts and inactivated human blood serum 1 part.

In preparing the above the Locke, Ringer's or normal salt solution is filtered and autoclaved at 15 pounds pressure for 15 minutes. The inactivated blood serum is added and the whole filtered through a Mandler or Berkefeld filter, tubed and kept in the incubator at 37°C. to determine sterility. If sterile, the media should be kept in the incubator at 37°C. until used.

Barret and Yarbrough's medium.—Barret and Yarbrough have succeeded in culturing *Balantidium coli* in vitro through eleven transplants over a period of 32 days. They used a medium composed of inactivated human blood serum and 0.5% salt solution the proportion of one part of serum to sixteen parts of salt solution. This medium is faintly alkaline to litmus. Eight-cc. quantities of medium were placed in tubes having a diameter of 10 mm. and a length of 150 mm., giving the medium a depth of about 100 mm. Inoculation of about 0.1 cc. of undiluted faeces containing mucus was made with a capillary pipette into the bottom of the tubes in order to insure the partial anaerobic conditions which favor the growth of the balantidia. The tubes were then incubated at 37°C. and subcultures were in general made every second day. Subsequent examinations showed that active balantidia were present only in the lower portion of the tube and that a moderate growth of bacteria as shown by a moderate clouding of the medium seemed to favor the balantidial growth.

Culture Media for Other Protozoa

N.N.N. medium.—This is used extensively for the cultivation of trypanosomes and *Leishmania*. With human trypanosomes, rat or human blood is substituted for the rabbit blood specified.

Cover 125 grams of chopped-up beef with 1000 cc. of water and place over night in the refrigerator. Strain and add 20 grams of peptone, 5 grams salt, 10 cc. of normal sodium carbonate solution and 20 to 25 grams agar. Prepare as for nutrient agar and sterilize. To 1 part of this one-quarter strength meat infusion nutrient agar, when melted and cooled down to 60°C., add twice its volume of defibrinated rabbit's blood. Under the designation N.N.N. medium (Nicolle Novy MacNeal) Nicolle has modified the medium so that there is only salt and agar in the base to which the blood is added, meat extract, peptone and sodium carbonate being omitted. To prepare this medium take 14 grams agar, 6 grams salt and 900 cc. water. Prepare as for ordinary agar, tube and sterilize. To 1 part of this medium liquefied and cooled to 48°C., add one-third its volume of defibrinated rabbit's blood. Mix thoroughly, slant, and allow to set. Use rubber stoppers or cover cotton plugs with melted paraffin to prevent evaporation of the condensation fluid. It is the Hb which seems essential in cultivating various blood protozoa.

Rogers used as a medium for *Leishmania* blood containing a small quantity of sterile 10% sodium citrate solution slightly acidified with citric acid. One or two cc. of the citrate solution were placed in the barrel of a syringe into which the splenic blood was aspirated directly. Incubation at 22°C. seemed essential to the development of the flagellated forms, higher temperatures shortening the period of growth or causing disintegration of the organisms. Other observers, having noted that certain organisms of this group develop well at 28°C., have used this characteristic as a basis for species differentiation.

Row's medium for Leishmania.—Take 10 cc. blood from rabbit's heart or arm vein of man, defibrinate the blood and then add 10 volumes of distilled water to lake the cells (liberation of Hb). One volume of this laked blood solution is added to two volumes of sterile 1.2% salt solution.

Bass' medium.—For the cultivation of malarial organisms, Bass takes from 10 to 20 cc. of blood from the malarial patient's vein in a centrifuge tube which contains 0.1 cc. of 50% glucose solution. A glass rod, or a piece of tubing, extending to the bottom of the centrifuge tube is used to defibrinate the blood. After centrifugalizing there should be at least one inch of serum above the cell sediment. The parasites develop in the upper cell layer, about $\frac{1}{50}$ to $\frac{1}{20}$ inch from the top. All of the parasites contained in the deeper-lying red cells die. To observe the development, red cells from this upper $\frac{1}{20}$-inch portion are drawn up with a capillary bulb pipette.

Should the cultivation of more than one generation be desired, the leukocyte (upper) layer must be carefully pipetted off, as the leukocytes immediately destroy the merozoites. Only the parasites within red cells escape phagocytosis. Sexual parasites are much more resistant. Bass thinks he observed parthenogenesis. The temperature should be from 40° to 41°C; and strict anaerobic conditions observed. Aestivo-autumnal organisms are more resistant than benign tertian ones. Dextrose seems to be an essential for the development of the parasites.

The Thompsons have rather simplified the method of Bass. They draw 10 cc. of blood into a test tube containing the usual amount of glucose solution. They then defibrinate the blood by stirring with a thick wire for about five minutes and remove the wire with the adhering clot. They then pour this defibrinated blood into several small sterile test tubes, which should contain at least a one-inch column. Rubber caps are adjusted over the cotton plugs and the tubes placed in the incubator.

Noguchi's medium (for treponemata).—Noguchi formerly first inoculated material containing treponemata into the testicle of rabbits, obtaining by this procedure a pure culture, after a few transfers to the testicles of other rabbits. He now grows the organism directly from serum from a chancre. Test tubes 2 by 20 cm. are filled with 15 cc. of a medium consisting of 2 parts of 2% slightly alkaline agar to which when melted and cooled down to 50°C. is added 1 part of ascitic or hydrocele fluid. At the bottom of the medium in the tube is placed a fragment of fresh sterile tissue, preferably a piece of rabbit's kidney or testicle. After the medium solidifies a layer of sterile paraffin oil is run in so that it covers the solid medium to a depth of 3 cm. in order to prevent evaporation. The material is inoculated at the bottom of the tube with a capillary pipette. Incubation at 37°C. is carried on for two weeks under anaerobic conditions. The tissue acts by removing any oxygen that may be present in the depths of the medium, anaerobiosis being a necessary condition. To obtain anaerobic conditions Noguchi uses an anaerobic jar in which these conditions are

produced by a combination of vacuum, hydrogen gas and pyrogallic acid. It is to be noted that many specimens of ascitic fluid are unsuited. The tubes of Noguchi and Bronfenbrenner are shown in Fig. 8. Bronfenbrenner uses a 1.5% agar instead of the 2% used by Noguchi.

M'Leod and Soga have simplified Noguchi's method as follows: Take a test tube and fit a perforated rubber stopper which can be pushed down the tube. A piece of glass tubing is passed through the stopper to project slightly into the test tube. The other end of the glass tube is drawn out into a capillary tube and bent over at an acute angle. The test tube is filled to ½ or ⅔ of its depth with neutral bouillon. This is freshly boiled and when cool a piece of sterile tissue is dropped in. A strip of sterile gauze is drawn through a glass bead and soaked in the material it is desired to culture and dropped into the bottom of the tube alongside the fragment of sterile tissue. Ascitic fluid is then run in to a point which would be reached by the bottom of the rubber stopper. As quickly as possible push in the stopper and when the fluid appears in the capillary tube seal off the end in a small flame. Material for study can be obtained afterwards by breaking off the capillary tip and introducing a capillary pipette.

Muhlens and Hoffman's serum agar (for treponemata).—Fill sterile test tubes one-third full with horse serum. This is sterilized on three successive days at 55°C. Then add an equal amount of a 3% agar containing 0.5% glucose which has been melted down and cooled to 50°C. The mixed serum agar is then kept at 55°C. for two hours. Such tubes are inoculated as for ascitic agar rabbit tissue media and incubated under anaerobic conditions, preferably in a flask from which the air has been exhausted and the remaining oxygen absorbed as shown in the anaerobic bottle described and illustrated in Fig. 12.

Noguchi's medium (for Leptospira).—Noguchi succeeded in cultivating on this medium *Leptospira icteroides*, the causative organism of yellow fever. It consists of one part of rabbit serum and three parts of Ringer's solution made semisolid with 0.3% agar and contained in tall tubes. One cc. citrated yellow fever blood is introduced into the lower part of the medium. A thin layer of liquid petrolatum is poured on the top of the medium, partial oxygen tension being necessary. The optimum temperature for growth is 33°C.

Kligler and Robertson have slightly modified this technique for cultivation of *Borrelia recurrentis*. They emphasize the importance of properly controlling the physico-chemical factors that act to limit and prevent growth and state that a balanced reaction can be secured by adding 1% peptone broth or egg albumin as buffer and covering the culture with a layer of oil. *B. recurrentis* is a strict aerobe and in order to permit adequate aeration the oil layer should not exceed 1.5 cm. in height. Ascitic fluid, horse or rabbit serum may be used as culture fluids. By using these methods Kligler and Robertson were able to cultivate this organism consistently from the blood of infected mice and rats, to maintain the viability of cultures for periods of at least three to seven weeks, and to carry them on in successive subcultures by transplanting at intervals of from two to four weeks.

For the cultivation of the *Borrelia* group Noguchi recommends ascitic fluid (without agar) plus fresh tissue, the selection of a suitable sample of ascitic fluid being an important factor. A suitable ascitic fluid causes a loose fibrin formation in the culture tube when mixed with the fresh tissue. The medium is covered with a thin layer of paraffin oil to hinder evaporation.

CHAPTER III

STAINING METHODS

I. For Bacteria

In order to study a bacterial or blood specimen the first essential is a properly prepared film; the matter of staining is of less importance.

The slide or cover glass, cleaned with soap and water or by a special solution, should be polished with a piece of old linen. If the glass surface has been freed of grease a loopful of water will spread out evenly over the entire surface. The only quick practical way to make a slide or cover glass grease-free is to burn the surface for a moment in a Bunsen or alcohol flame, care being taken not to warp a cover glass. To make a preparation, apply a very small loopful of distilled water on the slide or cover glass and, touching a colony with a platinum needle, stir the transferred culture into the loopful (not drop) of water. The mistake of taking up too much bacterial growth is almost invariably made. Fluid cultures do not need dilution. The mixture is smeared over a large part of the cover glass, or over an equal area of a slide, and it is allowed to dry. If very little water is used, the preparation dries readily. Otherwise it can be dried in the fingers high over a flame. As soon as dry, the cover glass should be passed three times through the flame, film side up, to fix the preparation. Slides also may be fixed by passing them five times through the flame, but the method by burning alcohol recommended for fixing blood films gives more satisfactory bacterial fixation. For routine work the stain recommended is a dilute carbol fuchsin. Drop about 5 to 10 drops of water on the cover glass, then add 1 drop of carbol fuchsin. Allow the dilute stain to act from one to two minutes, then wash in water, dry between small squares of filter paper (4 × 4 inches), and mount in balsam or in the oil used for the 1/12-inch immersion objective. Löffler's methylene blue is equally good as a stain. As a rule, better preparations are obtained with dilute stains than with more concentrated ones.

If, in the study of cells, granular differentiation is wanted, fixation with alcohol, osmic acid, etc., should be used. If only nuclei and cell outlines are desired, so that bacterial inclusions may be distinct against a perfectly clear back ground, fixation by flaming with alcohol is preferable, care being taken to burn off the alcohol immediately after flooding the preparation.

By far the best mounting medium is liquid petrolatum. This not only has the advantage of always being of proper consistence for mounts, as opposed to Canada balsam, which must frequently be made thinner with xylol, but it is less sticky and does not develop the acidity which causes balsam mounts of Romanowsky stains to fade. Furthermore, it has superior optical properties. It is also useful for mounting small insects and sporangia of moulds. For permanent preparations the border

of the cover glass should be sealed with gold size or some other cement. Some prefer to mount directly in water without preliminary drying. It is good practice always to keep the smeared side of the preparations up. By this simple rule, preparations can be carried through the most complicated staining methods without the necessity of using such expedients as scratching the cover glass, to see which is the film side. In grasping a cover glass with a Cornet or Stewart forceps, be sure that the tips are well inside the margin of the glass; otherwise the stain will drain off. In staining preparations on slides, the grease pencil and the glass tubing, as recommended under Blood Smears, will be found useful.

Löffler's Alkaline Methylene Blue.—Saturated alcoholic solution of methylene blue, 30 cc.; 1 to 10,000 caustic potash solution, 100 cc. (Two drops of a 10% solution KOH in 100 cc. of water makes a 1:10,000 solution.)

Carbol Fuchsin (Ziehl-Neelsen).—Saturated alcoholic solution basic fuchsin, 10 cc.; 5% aqueous solution carbolic acid, 100 cc.

Gram's Method.—This is the most important staining method in bacteriological technique. It is however likely to give unsatisfactory results in the hands of the inexperienced and in using it the following points must be kept in mind:

1. Laboratory cultures (subcultures) which have been carried over for years frequently lose their Gram characteristics.

2. Cultures which are several days old or dead or degenerated do not stain characteristically.

3. The aniline gentian violet deteriorates when exposed to light in two or three days—it should be kept in the dark. It should have a rich, creamy, violet appearance.

4. The iodine solution deteriorates and becomes light in color. It should be of a rich port-wine color.

5. Decolorization, best observed over a white background, should be stopped as soon as no more violet stain is seen to stream out from the preparation.

6. The preparation should be thin and evenly spread.

Stains and other reagents used:

1. *Gentian violet.*—(*a*) The original stain recommended was aniline gentian violet. This is made by mixing 1 part of saturated alcoholic solution of gentian violet with 3 parts of aniline oil water, which is prepared by adding 2 cc. aniline oil to 100 cc. distilled water, shaking violently for 3 to 5 minutes and then filtering several times to get rid of the objectionable oil droplets which, in a Gram-stained preparation, show as confusing black dots.

(*b*) Weigert has recommended the following stock solutions:

No. 1.		No. 2	
Gentian violet.....	2 Gm.	Gentian violet.....	2 Gm.
Aniline oil.....	9 cc.	Distilled water.....	100 cc.
Alcohol (95%).....	33 cc.		

These solutions keep indefinitely. To prepare the solution used in staining, mix 1 cc. of No. 1 with 9 cc. of No. 2 and filter. This keeps about two weeks.

(*c*) Carbol gentian violet is preferred by some to aniline gentian violet. It is made by adding 1 part of saturated alcoholic solution of gentian violet to 10 parts of 5% aqueous solution of carbolic acid. This has a tendency to overstain.

2. *Decolorizing agents.*—Alcohol 95% was originally recommended as the decolorizing agent, but acetone is preferred by many due to the fact that it is more rapid in its decolorizing action on Gram-negative organisms, yet slower to decolorize positive organisms. Acetone gives also a cleaner preparation than alcohol, particularly in smears of pus and sputum.

3. *Gram's iodine solution.*—The formula for this solution is:

Iodine	1 Gm.
Potassium iodide	2 Gm.
Distilled water	300 cc.

Sheppe and Constable demonstrated that when exposed to light and heat the iodine solution used in the Gram stain may become acid, owing to the formation of small amounts of hydriodic acid, which exercised a decolorizing effect on normally Gram positive bacteria. According to Kilduffe, sodium bicarbonate will neutralize the acid formed in the iodine solution as well as any that may be present in smears made from acid secretions.

His formula for the iodine solution is:

Iodine	1 Gm.
Potassium iodide	2 Gm.
Distilled water	240 cc.

When dissolved, add 60 cc. of 5% aqueous solution of sodium bicarbonate.

4. *Counterstains.*—Many counterstains have been used; the most commonly recommended are:

(*a*) Bismarck brown. This is prepared by dissolving 0.2 Gm. of the stain in 100 cc. of boiling water. It is then cooled and filtered.

(*b*) Diluted carbol fuchsin, made by mixing 1 part of carbol fuchsin with 10 parts of water.

(*c*) Safranin, made by mixing 10 cc. of a saturated alcoholic solution of water-soluble safranin with 90 cc. of distilled water. This stain keeps.

Technique of Gram staining:

The preparation to be stained should be thin and evenly spread. The film should be air-dried and passed through flame for fixing. The gentian violet is then applied and kept on for 2 to 5 minutes. Some hasten the staining by steaming as for tubercle bacilli. Next wash the preparation with water and then flood it with Gram's iodine solution. Some bacteriologists simply pour off excess of aniline gentian violet and immediately drop on the iodine solution. It is well to repeat the application of the iodine solution a second time. The iodine solution is left on one minute or until the preparation has the color of coffee grounds.

Wash off the excess of iodine solution at the tap, drop on 95% alcohol, or acetone, and decolorize until no more violet color streams out. Now wash again and counterstain with one of the stains mentioned above.

Gram-positive bacteria are stained a deep violet whereas the Gram-negative organisms take the color of the counterstain.

In staining smears of pus for gonococci or other Gram-negative bacteria it is best first to stain with the gentian-violet solution for two to five minutes. Then wash and examine the preparation mounted in water. The organisms stand out prominently. After noting the presence of the cocci treat the smear with the iodine solution and proceed as in the usual Gram staining technique.

Stirling's Gentian Violet.—Five grams of gentian violet are ground in a mortar with 10 cc. of 95% alcohol. After practical solution, 2 cc. of aniline oil are added and then 88 cc. of distilled water. The grinding is continued a short time and then the mixture is let stand a day or two when it is filtered through paper. It has the merit of staining quickly and intensely and of keeping many months.

The film, air-dried and passed through the flame, or preferably fixed in methyl alcohol, is stained as follows: The gentian violet is applied for one-half minute and washed or blotted off. The Gram's solution is applied also for one-half minute and after washing or blotting away the excess, the slide is immersed for a few seconds in a Coplin jar containing 95% alcohol. It is advisable to move it up and down. After washing away the alcohol, the counterstain, consisting of aqueous safranin, is put on for one-half to one minute or longer. It does not overstain.

Smith's Gram-eosin Stain.—This stain for sputum, devised by W. H. Smith, gives differential staining of the organisms present and shows cellular elements remarkably well.

Technique:

1. Make smear on cover slip and fix by heating very gently, the slip being held in the fingers.
2. Aniline gentian violet, bring to steaming point, wash with water.
3. Gram's iodine, bring to steaming point, wash with water.
4. Alcohol (95%) till no more color streams out.
5. Apply the stain, consisting of 0.5% aniline green in a 5% solution of (red) eosin in water; steam for five seconds; wash in water.
6. Löffler's alkaline methylene blue; bring to steam, wash with water.
7. Wash with absolute alcohol.
8. Clear stained smear with xylol; blot.
9. Mount in balsam or oil.

Gram-positive organisms are stained deep violet, almost black; Gram-negative organisms a light blue, and cells pink.

Gram Staining Reactions of Important Bacteria

Gram Negative	Gram Positive
Cocci	*Cocci*
Meningococcus	Staphylococcus group
Catarrhalis	Streptococcus group
Gonococcus	Pneumococcus group
	M. tetragenus

Bacilli

B. pneumoniae
B. pyocyaneus
B. influenzae
B. pertussis
B. fusiformis
B. coli group
B. typhosus group
B. dysenteriae group
B. mallei
B. tularensis
B. symptomatic anthrax
B. pestis
B. of Ducrey
B. of Morax-Axenfeld
B. of Koch-Weeks
B. melitensis

Spirilla

Cholera and allied forms
Mouth spirals

Bacilli

B. diphtheriae group
B. tetani
B. tuberculosis
B. leprae
B. anthracis
B. Welchii and other anaerobes

Spirilla

None important

Gram amphophil (variable)

Molds
Yeasts
Protozoa

Methods for Staining Acid-fast Bacilli. *Ziehl-Neelsen's method.*—1. Carbol fuchsin, with gentle steaming for three to five minutes, or in the cold for fifteen minutes. 2. Wash in water. 3. Decolorize in 95% alcohol containing 3% of hydrochloric acid (acid alcohol), until only a suggestion of pink remains. 4. Wash in water. 5. Counterstain in saturated aqueous solution of methylene blue or with Löffler's methylene blue. 6. Wash, dry, and mount.

The steaming of the slides with carbol fuchsin is most conveniently carried out by resting the slides on a piece of glass tubing bent into a V- or U-shape.

The following has been suggested for decolorizing and counterstaining the Bacillus tuberculosis:

20% lactic acid
Methylene-blue in excess.

At time of use, one part of the lactic-blue is added to four parts of 95% alcohol.

Effect of flaming.—Flaming immediately after flooding with alcohol causes cytoplasm to be unstained, the nucleus and bacteria appearing as enclosed in a clear ring.

The longer the alcohol is left in contact, the more completely will the cytoplasm take the stain. Hence, where only the presence of bacteria is to be determined, flame and stain (for instance, with dilute carbol fuchsin).

Leprosy bacillus differentiation.—The lepra bacillus is usually considered as being rather easily decolorized by alcohol. It is therefore often recommended to use 20%

aqueous solution of sulphuric acid or nitric acid for decolorization instead of the acid alcohol above recommended for tubercle bacilli. I have often found the leprosy bacilli as resistant to alcohol as tubercle bacilli.

Smegma bacillus differentiation.—The smegma bacillus is easily decolorized with acid alcohol and in a well decolorized smear from urinary sediment one can usually feel sure that any acid-fast bacilli are tubercle bacilli. I have had a case, however, where numerous smegma bacilli withstood decolorization for more than 20 minutes. Nikitin's solution (Glacial acetic acid, 66; 10% acetone in alcohol, 33) decolorizes smegma bacillus in 3 minutes, but leaves the tuberculosis bacilli stained for 10 to 15 minutes.

The standard method of differentiation is that of Pappenheim. After steaming in carbol fuchsin, pour off the stain and without washing treat film with the following: Corallin, 1 Gm.; absolute alcohol, 100 cc.; methylene blue, 0.66 Gm., and glycerin, 20 cc. The film is flooded several times with the mixture, which is allowed to drain off slowly following each application. Then wash in water and mount. The smegma bacillus is decolorized by the corallin and alcohol but the tubercle bacilli remain red.

Fontes' method.—Fontes has devised a method of staining bacilli normally acid-fast by which are stained the elements that have lost their acid fast properties wholly or in part as well as those that have retained them. By it are characteristically stained also the granules to which great importance has been attached by Much. The method is to stain the preparation with carbol fuchsin, decolorize with acid alcohol, then carry through the various steps of the Gram method, counterstaining, however, with Bismarck brown. Fontes in his method used 1 part of absolute alcohol and 2 parts of acetic acid as the decolorizing agent. I have obtained, however, just as satisfactory results with the acid alcohol. By this method the acid-fast tubercle bacilli show as red rods dotted with violet granules. Those which do not fully retain acid-fast properties show as zigzag violet lines.

Spengler's method.—This method is considered by many investigators as superior to all others in the staining of tubercle bacilli. The technique is carried out as follows:

1. Stain with carbol fuchsin, gently steaming for 3 to 5 minutes.
2. Pour off carbol fuchsin and apply picric-acid alcohol for 2 or 3 seconds. Picric-acid alcohol is prepared by dissolving 2 Gm. picric acid in 40 cc. distilled water; allow this to stand for 24 hours, then filter and add an equal volume of 96% alcohol.
3. Apply 3 or 4 drops of 15% nitric acid for 5 seconds.
4. Pour off nitric acid and apply picric-acid alcohol again until sputum looks yellowish.
5. Wash in water, dry and mount.

Schulte-Tigges method.—Flood slide with carbol fuchsin and heat to steaming for *one* minute. (Do not cook or boil.) Wash off excess stain and decolorize with 10% (freshly prepared)aqueous solution sodium sulphite. Wash thoroughly and counterstain with saturated aqueous solution of picric acid. Wash, dry and examine.

Smith's Formol Fuchsin.—This stain is made by dissolving 10 cc. each of saturated alcoholic solution of basic fuchsin, methyl alcohol and formalin in a sufficient quantity of distilled water to make a final product of 100 cc. It should not be used

until after standing twenty-four hours; it should then have a purplish tinge. This stain gives a very sharp differentiation of bacteria and nuclear structures. Fixation by heat gives the best staining. Allow stain to act for two to ten minutes. It is to be noted that after standing about two weeks the stain appears to lose its sharp staining power.

Archibald's Stain.—This is an excellent bacterial stain and has been highly recommended by Blue and McCoy in plague work.

Solution No. 1		Solution No. 2	
Thionin	0.5 Gm.	Methylene blue	0.5 Gm.
Phenol (crystals)	2.5 Gm.	Phenol (crystals)	2.5 Gm.
Formalin	1.0 cc.	Formalin	1.0 cc.
Water	100 cc.	Water	100 cc.

Prepare the two solutions separately and allow them to stand twenty-four hours. For use, mix in equal parts and filter. Smears, fixed by heat or otherwise, are stained for ten seconds.

Nicolle's Carbol Thionin.—To prepare this stain take 10 cc. of saturated solution of thionin in 50% alcohol and 100 cc. of 2% carbolic acid solution.

Pappenheim's Stain.—Take a very small portion of methylene green on the point of a penknife and shake it into a test tube; then take up twice as much pyronin and deposit it in the same test tube, and fill the test tube one-half full with water. The solution should have a distinct reddish-violet color, and a drop on a piece of filter paper show a violet center and peripheral green ring. The solution should be used fresh. Stain from two to five minutes. Differentiate with a little resorcin on a penknife point dissolved in a test tube one-quarter full of alcohol. Dehydrate, clear and mount. Polymorphonuclear nuclei stain greenish; nuclei of mononuclears and plasma cells from bluish-red to dull violet; cytoplasm of lymphocytes and plasma cells purplish-red and bacteria red.

A modification of this stain is as follows: Methylgreen, 0.15; pyronin, 0.5; alcohol (95%), 5.0; glycerin, 20; and 2% aqueous solution of carbolic acid to 100.0.

Romanowsky Stains.—See under section on Blood. For mounting specimens showing chromatin staining, as malarial parasites, trypanosomes, intestinal flagellates, etc., liquid petrolatum is to be highly recommended. With this, the chromatin staining lasts without fading for at least two years, whereas the acidity of balsam causes rapid fading of the chromatin.

Neisser's Stain for Diphtheria Bacilli.—This stain is made up in two solutions.

Solution No. 1	
Methylene blue	0.1 gram.
Alcohol	2 cc.
Glacial acetic acid	5 cc.
Distilled water	95 cc.

Dissolve the methylene blue in the alcohol and add it to the acetic acid-water mixture. Filter.

Solution No. 2	
Bismarck brown	0.2 gram.
Water (boiling)	100 cc.

Dissolve the stain in the boiling water and filter.

To stain: Fix the preparation. Pour on the dilute acetic acid-methylene blue solution and allow to act from thirty to sixty seconds. Wash. Then pour on the Bismarck brown solution, and after thirty seconds wash off with water. Dry and mount. The bodies of the bacilli are brown with dark blue dots at either end.

Neisser recommends only five seconds as the time of application of each solution. He also recommends that the culture be only nine to eighteen hours old and that the temperature of the incubator shall not exceed 36°C. Incubation at 37°C. gives satisfactory results, however.

Ponder's Stain for Diphtheria Bacilli.—This stain is made according to the following formula:

Toluidin blue (Grubler)	0.02 gram.
Glacial acetic acid	1 cc.
Absolute alcohol	2 cc.
Distilled water to	100 cc.

The film is made on a cover glass and fixed in the usual way. A small quantity of the stain is spread on the film and the cover glass is turned over and mounted as a hanging-drop preparation. Diphtheria bacilli are recognized by metachromatic granules stained with striking intensity, and diphtheroids, by their more intense staining, are sharply differentiated from ordinary cocci and bacilli which show in the preparation only as faint light-blue bodies. It is a most excellent stain for bringing out the ascospores of yeasts. In my opinion this stain is more valuable than the Neisser.

Laybourn's Modification of Albert's Stain for Diphtheria Bacilli.—By substitution of malachite-green for methyl-green of Albert's formula the staining of both granules and body of cell is deepened without destroying the marked contrast between these elements. Albert's formula as modified by Laybourn:

Solution 1	Toluidin blue	0.15 Gm.
	Malachite-green	0.20 Gm.
	Glacial acetic acid	1 cc.
	Alcohol, 95%	2 cc.
	Distilled water	100 cc.

Let stand 24 hours filter, use.

Solution 2	Iodin crystals	2 Gm.
	Potassium iodide	3 Gm.
	Distilled water	300 cc.

Technique:

1. Fix smears by heat.
2. Solution 1, 3–5 minutes.
3. Wash in water.
4. Solution 2, one minute.
5. Wash with water: blot dry.

The granules of diphtheria bacilli stain black, the bars dark green, and the intermediate portions a light green. The contrast is marked. Stain is serviceable in detecting diphtheria bacilli when there are very few.

Capsule Staining.—It may be noted that the best method for studying bacteria, as to presence of capsules, is in the hanging drop, with the greater part of the light shut off by the diaphragm.

Gram's method.—In material where capsules are well developed, as in pneumonic sputum, the Gram method of staining brings out the capsule perfectly. This is of diagnostic value, as the more or less nonpathogenic pneumococci common about the mouth do not seem to show a capsule when stained in this way.

The India-ink method of staining gives good results for capsules.

Welch's glacial acetic acid method.—(1) Cover the preparation with glacial acetic acid for a few seconds; (2) drain off and replace with anilin gentian-violet solution; this is to be repeatedly added until all the acid is replaced; (3) wash in 1 or 2% solution of sodium chloride and mount in the same. Do not use water at any stage. The capsule stains a pale violet.

Huntoon's method.—A 3% solution of distilled water of nutrose (sodium casinate) is cooked for one hour in an Arnold sterilizer and tubed unfiltered after adding 0.5% phenol. The organisms to be stained are mixed with a drop of this solution of nutrose, spread in thin film on a glass slide and dried in air, not fixed. The stain is as follows: phenol (2% aqueous solution), 100 cc.; acetic acid; (1% aqueous solution), 1 cc.; lactic acid (concentrated), 0.5 cc.; carbol fuchsin, 1 cc.; basic fuchsin (saturated alcoholic solution), 1 cc. The film is stained for thirty seconds, washed in water and dried.

Hiss' capsule stain.—Preparations are best made by direct films from pneumococcus exudates. Dry in air and fix by heat. Stain for a few seconds with saturated alcoholic solution of fuchsin or gentian violet, 5 cc., in distilled water, 95 cc. Flood the slide with the dye and hold the preparation for a second over a free flame until it steams. Wash off the dye with 20% aqueous copper sulphate solution. Blot (do not wash in water).

By this method the capsule appears as a faint blue halo around a dark purple cell body. Better results may frequently be obtained by omitting heat fixation and by washing off the dye with the copper sulphate solution as soon as it begins to steam. Water should not be applied at any stage of the procedure.

Wadsworth's method.—In this technique the capsule staining depends on fixation with a good quality formalin for about 3 minutes. After fixation wash in water for 3 or 4 seconds only. Then stain as usual by any simple aniline dye or by Gram.

Flagella Staining.—Inoculate a tube of sterile water (gently) in upper part, with just enough of an eighteen to twenty-four-hour-old agar culture to produce faint turbidity. Incubate for two hours at 37°C. From the upper part of the culture take a loopful and deposit it on a cover glass. Dry in thermostat for one to five hours or over night. Perfectly clean cover glasses must be used, and all manipulations should be performed gently to avoid breaking off flagella. The following two methods are the ones most commonly used.

Plimmer and Paine method has proved satisfactory even in the hands of beginners. The stain is made as follows:

Tannic acid,	10	Gms.
Aluminum chloride (hydrated),	18	Gms.
Zinc chloride,	10	Gms.
Rosaniline hydrochloride,	1.5	Gm.
Alcohol 60%,	40	cc.

The solids are placed together in a mortar, and at once, (i.e., before deliquescence) triturated with the alcohol. 10 cc. of alcohol are used first and the mass is mixed thoroughly, care being taken to smash up the whole of the zinc chloride—at this stage a homogeneous paste of a golden brown colour is obtained—the rest of the alcohol is then stirred in slowly, when the mass goes gradually into a viscous solution of deep red colour. In this state the stain appears to remain stable for several years. For use it is diluted with water when nearly complete precipitation occurs, a small amount remaining in solution, evidently as a balanced colloid, which, when applied to the bacterial film, becomes adsorbed upon the flagella and periphery of the organism. No further treatment is necessary, but it is advisable to stain the body of the organism with fuchsin or methylene blue.

Method of application of the stain:

(1) The culture must be of suitable age; eighteen to twenty-hour cultures at 20°C. usually prove satisfactory. The growth is removed and, with as little disturbance as possible, stirred into water to give a distinctly turbid suspension.

(2) As in all methods for staining flagella, it is essential that the slides be scrupulously clean. Cleaning is best done with chromic acid, and the last traces of grease are got rid of by roasting the slides strongly on a wire gauze over a bunsen flame.

(3) The slide is allowed to cool to about blood heat, and a drop of the suspension is placed at one end of the slide with a 3 mm. loop. The slide is at once tilted so that the drop runs down the slide. It seems to be very important that the film should dry quickly, hence the use of a warm slide. As an alternative method, the drop may be drawn across the slide by means of a strip of guttapercha tissue. Some excellent preparations have been obtained in this way.

(4) The mordant is applied to the film without fixing as follows: 1 part of the stain, (0.5 cc. say) is mixed with 4 parts of water, (2 cc.) in a small corked specimen tube. After inverting the tube three or four times it is allowed to stand for sixty seconds, then filtered through a small filter on to the slide and again allowed to remain for sixty seconds, when a slight bronzing should be visible on the surface. It is then washed rapidly under the tap. (A certain amount of license with the time, up to two or even three minutes, may not be disastrous, but it is better to adhere rigidly to the times as given.)

(5) The film is then flooded with cold carbol fuchsin for five minutes, washed, dried, and examined in oil. If satisfactory, the film may be mounted in canada balsam, or better in euparal, and no fading of the stain seems to occur.

Zettnow's method.—Dissolve 10 Gm. tannin in 200 cc. water, warm to 50° or 60°C., and add 30 cc. of a 5% aqueous solution of tartar emetic. The turbidity of the mordant should entirely clear up on heating. The mordant should keep for months when a small crystal of thymol is added to it.

Next dissolve 1 gram silver sulphate in 250 cc. distilled water. Of this solution take 50 cc. and add to it drop by drop ethylamine (this comes in a 33% solution) until the yellowish-brown precipitate which forms at first is entirely dissolved and the fluid is clear. Only a few drops are required. The bacterial preparations prepared as described above are floated in a little mordant contained in a Petri dish and heated over a water bath for five to seven minutes. Take the dish containing the preparation off the water bath and, as soon as the mordant becomes slightly opalescent as the result of cooling, remove the cover-glass preparation and wash thoroughly in water. Then heat a few drops of the ethylamine silver solution upon the mordanted cover preparation until it just steams and the margin appears black. Next wash thoroughly in water and mount. This gives the most satisfactory results of any method with which I have ever experimented.

Spore Staining.—The most satisfactory method of demonstrating spores is by staining bacterial preparations with dilute carbol fuchsin or Löffler's methylene blue when the nonstaining spores appear as highly refractile pieces of glass in a colored frame.

The acid-fast method, as used for tubercle bacilli, gives good results. The decolorizing must however, be lightly done; otherwise the spore will lose its red stain.

Möller's method.—Fix films and then treat with chloroform for one or two minutes. Wash thoroughly and treat with a 5% solution chromic acid for one minute. Wash in water and then stain as for acid-fast organisms with carbol fuchsin, using a 1% sulphuric acid solution instead of the 3% acid alcohol.

Abbott's method.—This method gives a beautiful picture. Cover fixed film with Löffler's alkaline methylene blue, heating the preparation to boiling from time to time—not continuously. Keep this up one minute. Wash in water and then decolorize in 2% nitric acid alcohol until the blue disappears. Wash and apply for a few seconds an eosin solution (sat. alc. sol. eosin 10 cc., water 90 cc.).

Wash, dry and mount. The spores are stained blue.

Agar Jelly Staining Method of H. C. Ross.—Very clear 1.5% solution of agar is colored with Unna's polychrome methylene blue, Giemsa's solution, thionin or

Gram's solution of iodine. Very thin smears of blood, faeces or gastric content sediment are made and either fixed lightly in the flame or air-dried. A drop of the melted, colored agar solution is placed on the smeared cover glass and this is mounted immediately on a clean slide. The preparation is ready for examination in about two minutes.

Intravital Staining.—A perfectly clean slide is flooded with a saturated aqueous solution of methylene blue or 10% toluidin blue to form an even film. When dry the slide has a clean blue color. To stain organisms deposit a loop of the culture, or material emulsified in saline, on a cover glass. Mount on the stained slide. The organisms take up the stain.

II. For Protozoa

For methods of staining blood protozoa, see chapter XIII.

Unless dealing with albuminous material it is well to add a little blood serum, albumin fixative or white of egg to the preparation—about one loopful to a smear. A stock of serum or white of egg is best preserved by the addition of 2% chloroform and kept tightly corked.

Fixation of Amoebae.—Carnoy's fixative, which consists of absolute alcohol 6 parts, chloroform 3 parts, glacial acetic acid 1 part is reported by French as excellent for any staining methods. It is especially useful when followed by haematoxylin. Used in the cold, insures quick killing, rapid fixation, and fidelity of tissue elements when followed by haematoxylin stains. Immerse moist smear in fixative for 10 to 12 minutes, wash in absolute alcohol (no water) 10 to 15 minutes, in 95% alcohol 10 to 20 minutes, and then in distilled water 10 to 20 minutes. Stain.

Fixation of Intestinal Flagellates.—Nöller's method is to fix in warm concentrated bichloride of mercury solution 10 to 30 minutes, wash in gently running water for 10 minutes, drain but do not allow to dry, wash with physiologic salt solution and immerse in clear sterile serum (e.g. horse) for 5 to 15 minutes. Remove and allow to dry, wiping off back of slide first. Fix in absolute alcohol for 10 to 20 minutes, allow to dry and stain as an ordinary blood film.

Giemsa's Method.—Fix moist smears by immersion for 1 to 12 hours in a fixative made by adding 1 part of 95% alcohol to 2 parts of saturated aqueous solution of bichloride of mercury. Wash for a few seconds in water and then for about five minutes with a dilute Lugol's solution (KI, 2 Gm.; Lugol's solution, 3 cc.; water, 100 cc.). Wash in water and then in a 0.5% solution of sodium thiosulphate to remove the iodine which was used to remove the mercury. Wash in water five minutes, then stain with Giemsa's stain as used in blood work for one to ten hours. Wash and mount.

Vital Staining with Neutral Red Solution.—As a stock solution one uses a 0.5% aqueous solution of neutral red. Protozoa take a rose-pink color with a distinct differentiation between endoplasm and ectoplasm, but should the faeces be quite alkaline the neutral red will be decomposed with the formation of bilirubin-like crystals.

Panoptic Method.—Highly to be recommended for the staining of protozoa, whether in smears or in sections, is the panoptic method.

1. Wright's or Leishman's stain for one minute.

2. Dilute with water and allow dilute stain to act for three to ten minutes. Wash in water and then

3. Pour on dilute Giemsa's stain. Allow to stain from thirty minutes to twenty-four hours. Differentiate with 1:1000 acetic acid solution until blue stain just shows commencing diffusion into the acetic acid. Then wash in water, 95% alcohol, and absolute alcohol; treat with xylol and mount in liquid petrolatum.

With preparations other than blood smears, as sections, it is better to go from 95% alcohol to oil of origanum, then mount.

Owing to the great value of a sharp nuclear picture in differentiating amoebae it is of great importance to use some iron haematoxylin method as that of Heidenhain described below.

Mallory's Phosphotungstic Haematoxylin.—Fix moist smears, film surface down, in Zenker's fluid for five to ten minutes. Wash in water, treat with Gram's solution and wash with 70% alcohol until all the yellow color is discharged. Wash in water. Then stain with *Mallory's phosphotungstic haematoxylin* for one-half hour. Wash, clear and mount. See appendix.

Mallory's Differential Stain for Amoebae.—Stain in saturated aqueous solution of thionin for from three to five minutes. Then wash in water, clear and mount. Nuclei of amoebae are stained a brownish-red.

Iodine-Eosin Stain for Amoebae.—Kofoid's modification of Donaldson's stain has given us very satisfactory results. Mix equal parts of (1) saturated solution of eosin in normal saline (2) 5% solution of potassium iodide in normal saline saturated with iodine. This mixture when used should not be more than twelve hours old.

For examination emulsify a particle of the faeces in a small drop of saline and alongside of this a similar emulsion in the iodine-eosin stain. Cover the two drops with one cover glass. In the stained area the bacteria, faecal particles and the intestinal yeasts (except the larger forms) stain at once. Against the pink background the protozoan cysts stand out clearly as bright spherules which soon become tinged with the iodine to varying tones of yellow, while their glycogen-filled vacuoles, when present, turn light or dark brown according to their mass. The nuclei become more clearly defined as the iodine penetrates, especially in *E. coli* and *E. histolytica;* they are detected with difficulty in this stain in *E. nana.*

Heidenhain's Alcoholic Iron Haematoxylin.—1. Fix for 15 minutes in Schaudinn's solution. (Two parts of saturated aqueous solution of corrosive sublimate, 1 part of absolute alcohol.)

2. Place at once in 70% alcohol for at least one hour.

3. Place for five hours or longer in the following mordant: 50% alcohol, 10 parts, 4% aqueous iron alum solution, 1 part.

4. Stain for 12 to 24 hours in the following solution: Heidenhain's haematoxylin, 1 part and 70% alcohol, 10 parts.

Heidenhain's haematoxylin is prepared by dissolving 1 Gm. haematoxylin in 10 cc. absolute alcohol and then adding 90 cc. distilled water and a crystal of thymol.

5. Differentiate in the solution used as mordant in (3).

6. Wash thoroughly in several changes of 70% alcohol to remove mordant.

7. Dehydrate and mount in Canada balsam.

Mallory's Iron Haematoxylin Method.—I have obtained beautiful staining with this simple method. The great point in technique is the watching of the differentiation.

Treat sections or moist smears, fixed by Giemsa's method, with 10% aqueous solution of ferric chloride for three to five minutes. Then drain off iron solution, blot the section and stain for four minutes with a freshly made solution of 1% haematoxylin in water. To make this add a few small crystals to 4 or 5 cc. water in a test tube and dissolve by heat. The stain deteriorates after twenty-four hours. Wash in water. Differentiate with a 0.25% aqueous solution of ferric chloride. The differentiation is complete in from a few seconds to one or more minutes according to depth of staining and thickness of film. Wash in water, pass through alcohols and xylol and mount.

Fontana's Method (*for treponemata*).—To obtain serum for examination for treponemata, wash the lesion with alcohol; dry, and rub with gauze, or gently scrape with a scalpel, so that lymph from the corium may be obtained. Blood should be gently wiped away and the specimen made from the lymph that eventually exudes clear. The serum may also be obtained by washing the lesion, and, while the surrounding surface is still wet, applying a Bier cup or a test tube in which a partial vacuum has been created by flaming. The smears should be thin, and spread on scrupulously clean slides.

Tribondeau's modification of the original Fontana method is here described. Cover the dried smear repeatedly during about a minute with *Ruge's solution:*

Pure acetic acid	1 cc.
Formalin, (40%)	2 cc.
Distilled water	100 cc.

To complete fixation alcohol is dropped on the slide and then flamed. The following mordant is then applied:

Tannic acid	5 Gm.
Distilled water	100 cc.

Warm gently till steam rises and then allow to act for 30 seconds longer. Wash slide with distilled water for a few seconds and then cover slide with Fontana's solution. (To 5% silver nitrate solution ammonia is added, drop by drop, with a capillary pipette, till a sepia precipitate forms and redissolves. To this solution more silver nitrate is added till a solution is produced which remains slightly cloudy on shaking.) Cover slide with this solution and warm gently till steam arises; allow the solution to act for 30 seconds longer. Treponemata appear dark brown to black but fade in a few days under cedar oil or Canada balsam. The use of carbol fuchsin has been recommended in place of Fontana's silver solution.

We have used the highly recommended Hollande stain but find the above method superior.

Benian's method. (*Udasco's modification*).—Mix on a cover-glass one or two loopfuls of 2% aqueous solution of Congo red with a small amount of the serum or exudate from the lesion. Spread this mixture evenly and thinly. *Dry in air.*

Fume over concentrated HCl, until the film is a greenish-blue. Do not wash at any time. The treponemata appear white and unstained on a homogeneous blue ground. This method may be used on dried preparations, first treating the film with the Congo red.

Tilden's Method (*for treponemata*).—This method is recommended by Noguchi. The following solution is used as a fixative:

Phosphate buffer solution (pH 7.6) (*M*/15 Na_2HPO_4 88 *cc. and M*/15 KH_2PO_4 12 *cc.*)	90 cc.
Formaldehyde solution	10 cc.

Tissue scrapings are suspended in a small amount of this solution and the mixture allowed to stand for at least five minutes. The longer the fixation the better the results; the organisms remain well preserved for at least two or three weeks. Prepare thin films from this mixture on clean slides and dry in air. If the amount of material available is very small, a drop of the fixative may be put on a slide and a drop of exudate added. Let stand for five minutes, protected from evaporation, and then spread out in a very thin film and dry in air. The film surface is flooded with a saturated alcoholic solution of gentian violet, or fuchsin, or with Stirling's solution of gentian violet such as is used for Gram's stain. The slide is almost immediately washed in running water and air-dried.

Warthin and Starry's method (*for staining treponemata*).—Maintaining that morphological differentiation of treponemata by the dark-field is often impossible, that no simple method of staining treponemata is known and that the India-ink method may be dangerously misleading, they urge the use of silver-impregnation methods and present their technique of demonstrating treponemata in cover-glass smears as the surest and safest diagnostic method yet devised.

The treponemata in the smears appear black against a light background. Since there takes place a deposition of silver on the surface of the organisms, their apparent size is increased, but morphological characteristics are not obscured.

Method: Note that all reagents must be chemically pure and that clean porcelain or glassware exclusively should be used.

1. Prepare smears on perfectly clean No. 1 cover glasses.
2. Dry thoroughly in air.
3. Fix in absolute alcohol 3–5 minutes.
4. Wash in distilled water, two changes.

If it is desirable to clear a too opaque background, the smear at this stage is immersed in concentrated hydrogen peroxide for 5–20 minutes and then washed thoroughly in distilled water.

5. Rinse the cover glass bearing the smear in 2% silver nitrate, and apply to its smear-side a perfectly clean cover glass also rinsed in the silver nitrate solution. Place the apposed cover glasses, setting them carefully on edge and in such manner as to avoid separating them, in a wide-mouthed dark bottle containing enough 2% silver nitrate for the level of the fluid to come about half way up the cover glasses. Place in incubator for 1–2 hours. Then remove cover glasses and separate them.

6. Place the cover glass bearing the smear, smear-side up, in the reducing solution for 30 seconds to 2 minutes.

Dissolve in order

2% silver nitrate solution	3 cc.
Warm 10% aq. gelatin solution	5 cc.
Warm glycerin	5 cc.

Stir in

Warm 1.5% agar suspension	5 cc.

Just before using, add, stirring vigorously,

5% aq. hydroquinone solution	2 cc.

The agar suspension is made as follows: Break up 1.5 grams of agar and soak in 20–30 cc. of distilled water until saturated. Pour off water and wash the agar with several changes of distilled water. Add 100 cc. distilled water, and bring to boiling point, stirring constantly. Allow to cool, shaking occasionally, and finally, when agar begins to set, shake violently to break it up thoroughly. Placed on a paraffin oven, the suspension remains just fluid enough to run.

7. When the reduction is complete, remove smear and rinse in 5% sodium thiosulphate solution.

8. Rinse in distilled water.

9. Absolute alcohol, xylol, balsam.

If the stained smears are to be preserved they may, following steps 8, be toned in:

Ammonium sulphocyanide	6.25 Gm.
Tartaric or citric acid	0.50 Gm.
Sodium chloride	1.25 Gm.
Distilled water	250.00 cc.
Solution of gold chloride (1:100)	6.25 cc.

After a short time in this solution (5–15 minutes) the stain turns to a blue-black color. The smears are then washed in distilled water, dehydrated and cleared, and mounted in balsam.

CHAPTER IV

STUDY AND IDENTIFICATION OF BACTERIA—GENERAL CONSIDERATIONS

In order to study bacteria it is necessary to isolate them in pure culture. This may be accomplished by taking one or more loopfuls of the material and mixing it in a tube of melted agar or gelatin. From this first tube one or more loopfuls are transferred to a second tube of melted agar or gelatin, and from this a third transfer is made, thereby giving tubes in which the distribution of the bacteria is one or more hundred times less in the second than in the first tube, and equally more dilute in the third than in the second. When the contents of the tubes are poured into Petri dishes, the bacterial colonies on the first plate are so thick that it would be impossible to pick up a single colony with a platinum needle without touching an adjacent one. On the second plate the distribution might be such that there would be discrete, well separated colonies, material from which could be taken up on the point of the needle or loop without touching any other colony. If the second plate did not meet these requirements, the third would.

In clinical bacteriology we work almost entirely with organisms preferring blood-heat temperature, hence it is necessary to use agar or blood agar as standard media for the obtaining of isolated colonies. Gelatin is of little value for this purpose in medical work. In using agar it will be remembered that it solidifies at a temperature slightly below 40°C. and does not melt again until it is subjected to a temperature practically that of boiling. Further, if the temperature of the media exceeds 44°C. it may affect injuriously the organisms we wish to study. Consequently it requires careful attention and quick work to inoculate the tubes, mix the contents, transfer and pour into plates within a temperature range not injurious to the organisms, and not inducing the solidification of the agar.

Deep and Superficial Colonies.—Colonies developing from organisms fixed at the surface of the agar at the moment it sets, and the more numerous colonies developing from bacteria caught in the depths of the media, are termed respectively *superficial* and *deep*.

At times great difficulty is experienced in determining whether a colony is deep or superficial; but, except to the person of large experience, greater difficulty, in fact

the greatest arising in the study of bacteriology, is met when trying to distinguish differences in deep colonies. These difficulties may be evaded by using the method of simply stroking plates along five or six parallel lines from one side of the plate to the other with a bent glass rod, platinum loop, or a small cotton swab, by which are obtained colonies which are well separated and entirely superficial. For this purpose poured plates of agar, blood agar or Endo media are kept on hand in the refrigerator.

Barber's Technique for Isolating a Single Bacterial Cell.—This method is of the greatest assistance in securing indisputably pure cultures of bacteria, especially anaerobic spore bearers.

The method is carried out as follows: A glass box about 7 × 3 × 2 cm., open at one side and without a top, is fixed in the mechanical stage of a microscope. A large cover slip, 60 × 35 mm., is lightly coated with sterilized petrolatum, and a drop of the culture, diluted if necessary, deposited on its greased surface. The cover slip is then placed across the open top of the glass box, greased side down. The lens is brought into a focus on the drop of culture which is then moved to one side, the objective remaining in place. A very firm capillary pipette bent at right angles, with the tip so drawn out as to present a lumen not more than 0.5 mm. in diameter, and with the other end attached to a delicate rubber tube for making suction, is introduced through the open end of the box and the tip brought into focus. The pipette may be guided by hand if one is very skillful, or a mechanical device may be employed.

The culture drop is now moved back to come into contact with the pipette, and capillary attraction draws in a few organisms. Again moving the mechanical stage, the material is blown out on another portion of the slip and little lakes form, one of which may contain a single organism. Taking a fresh sterile pipette, one end is applied to the lake selected as containing one organism, and the fluid drawn into the pipette. Thus a solitary organism is secured for transference to culture media or for injection into an animal.

Smeared or Stroked Petri Plates.—The material, as pus, faeces, throat membrane, etc., should be evenly distributed in a tube of sterile saline or bouillon, the swab which was originally used for obtaining the material being then pressed against the sides of the test tube to express excess of fluid and then stroked gently over successive lines on one plate; or, if the organisms be very abundant, over a second plate without recharging it from the inoculated tube. With such material as that adhering to the loop of a nichrome wire applicator which has been scraped along the posterior pharynx of a meningococcus carrier, it is satisfactory simply to touch the surface of the blood agar plate, to deposit some of the mucoid material, then, with a bent glass rod, spreading it over the entire surface of the plate, revolving the plate while drawing back and forth the smearing rod.

A nichrome wire loop, somewhat bent over and having a flattened-out loop end, the weight of the handle of the nichrome wire being supported by the fingers so as not to exert pressure and cut the surface of the medium, makes a good spreader.

According to my experience a very satisfactory method is to take a loopful from the bouillon tube suspension of the infected material and deposit it on the left half of the plate; then, without recharging the loop, we touch the right half of the

plate. Now taking a bent glass rod from a jar of 95% alcohol, we flame it and, to cool it, press the bent portion into the middle of the plate. This also divides the surface of the plate into two portions. Then rubbing the bent rod over the smaller amount of the material on the right side we carry it over the entire right side. Then go to the loopful deposited on the left side with the rod and rub it over this side.

FIG. 14.—Agar plate streaked by method described on p. 71.

For urine, deposit 1 drop on one side and 5 drops on the other; urine from a case of colon cystitis or pus kidney, or from a carrier of enteric organisms, however, should be diluted. A smear from pus, sputum, urine or throat culture should always be made before plating out in order to get an idea as to the degree of dilution required.

We use blood agar plates as routine ones, when we culture from throat, glands, joints, pus, blood, etc.; their lack of translucency not interfering when we study the morphology of superficial colonies with a hand lens. For demonstrating the haemolytic zone of *S. pyogenes* or the green of *S. viridans* or of the *Pneumococcus* such a medium is indis-

pensable. All pathogens grow well on it. For faeces we use Teague or Endo medium.

Esmarch's Roll Culture Tubes.—Having melted about 5 cc. agar in a test tube we inoculate the melted medium at 45°C. and very quickly roll the tube in a groove melted out of a block of ice. The agar sets on the sides of the tube and colonies may be studied with a glass. Such tubes form a large amount of water of condensation which aids in the study of streptococci. Larger tubes with rolled media are useful for culturing bacterial growth for vaccines, this technique giving a larger surface than the slant.

Slants.—To obtain isolated colonies on blood-serum or blood-streaked agar, which can be touched and by transfer obtained in pure culture, we simply smear the material on a slant of either medium. Then, without sterilizing the loop, we smear it thoroughly over a second slant, and so on to a third, or possibly a fourth or fifth.

An excellent method of colony isolation is to inoculate the water of condensation of an agar or blood agar slant with the material to be examined. Then without recharging we inoculate the water of condensation of a second slant and then on to a third or fourth tube. After the inoculation of the condensation water of the several tubes is completed allow the fluid to flow over the slant of each tube and then put tubes in the incubator. The colonies which develop on the slant are easily touched by the platinum needle to make transfers (fished). Colonies on such slants can be studied with a lens or with a low power objective. To determine the number of slants necessary for satisfactory separation of colonies, examine the material to ascertain the abundance of organisms before beginning the inoculation. It should be noted that the water of condensation, following incubation, makes a most satisfactory material for hanging-drop examination.

Stock Cultures.—The proper maintenance of stock cultures is an important item in a well equipped laboratory. For this purpose stab cultures are preferable to slants as they require much less frequent transfer and are less easily damaged if packed for shipment.

Classification.—At present the classification of the bacteria is very unsatisfactory from a scientific standpoint. The nomenclature abounds in instances where three or four terms are used in naming a single bacterium, instead of the single generic name and single specific one as is used in zoölogical nomenclature. This matter of nomenclature is, however, a subordinate factor in the confusion, since, when we begin to investigate, we find that different names have been applied to organisms apparently identical.

The slightest variation in morphological, locomotor, or biological characteristics seems to be considered sufficient grounds by many observers to justify the description of a new species, and, of course, the giving of a new name. Many of these names of doubtful validity but still retained, were applied prior to the epoch-making introduction of gelatin media by Koch (1881) and consequently at a time when the isolation of organisms in pure culture was a matter of extreme difficulty and uncertainty.

In the text to follow, the term bacterium has been used as a general designation for all schizomycetes. Migula calls motile rod-shaped organisms bacilli, and nonmotile ones bacteria. Lehmann and Neumann call spore-bearing organisms bacilli, and nonspore-bearing ones bacteria.

The *B. typhosus* is very motile and does not possess spores. According to Migula, it would be the *Bacillus typhosus;* according to Lehmann and Neumann, the *Bacterium typhosum.* The *B. anthracis* has spores and is nonmotile. Hence it would be *Bacterium anthracis,* according to Migula, and *Bacillus anthracis,* according to Lehmann and Neumann.

In the use of the keys at the head of each group of organisms it will be observed that the primary separation is on the basis of morphology—the cocci in one group, the bacilli in three subgroups, one for those rod-shaped organisms showing branching and curving forms, one for the spore bearers and one for the simple rods. The spirilla are grouped by themselves.

In this edition the nomenclature as given in Bergey's Determinative Bacteriology has been followed in the bacteriological keys, the older designations having been retained in the accompanying text.

In a study of the subject of bacterial classification by the Society of American Bacteriologists the general class designation of *Schizomycetes* is recommended. Six orders are recognized, only two of which, (excluding *Spirochætales*) the *Actinomycetales* and *Eubacteriales,* contain the organisms ordinarily encountered in medical bacteriology. In the first of these there are two families, *Actinomycetaceae* including the *Actinomyces bovis* and *Leptotrichia buccalis,* and the *Mycobacteriaceae* including the organisms of tuberculosis (*Mycobacterium*), diphtheria (*Corynebacterium*) and glanders (*Pfeifferella*). There are five families in the order *Eubacteriales,* four of which are of medical interest, namely the *Coccaceae,* the *Spirillaceae,* the *Bacillaceae* and the *Bacteriaceae.* The *Coccaceae* include all round organisms. The following type species for the composing genera may be given; *Neisseria gonorrhoeae, Streptococcus pyogenes, Staphylococcus aureus, Micrococcus luteus* and *Sarcina ventriculi.* Under the family *Spirillaceae* we have the genera *Vibrio* (type species *V. comma*) and *Spirillum* (type species *S. undula*). The *Bacillaceae* are rod-shaped organisms, producing endospores and have two genera: *Bacillus* and *Clostridium.* The genus *Bacillus* (type species *B. subtilis*) is aerobic and the rods are not much changed at sporulation; the genus *Clostridium* (type species *C. butyricum*) is anaerobic and the rods enlarge at sporulation forming clostridium forms. The *Bacteriaceae* are nonspore-bearing, rod-shaped organisms and include eleven tribes, the most important of which are the *Hemophileae* (type species *Hemophilus influenzae*), *Pasteurelleae* (*Pasteurella avicida*), *Lactobacilleae* (*Lactobacillus caucasicus*) and *Bacterieae* (*Escherichia coli* and *Eberthella typhi*).

Motility.—One of the first facts noted by the student in taking up bacteriology is the difficulty in determining motility, it being easily confused with Brownian movement, a sort of scintillating motion of

particles, and that due to currents in the preparation which is characterized by the swarming of all the bacteria in the same direction, going very fast at times, and then more slowly.

If in great doubt, the mounting of the organisms in a 2% solution of carbolic acid will stop movement if it be true functional motility, while Brownian and current movements are not interfered with. If truly motile, bacteria move in opposite and in all directions, and move away from the place where first observed unless degenerated or dead.

At times we base our assertion of motility on the presence of this characteristic in a few of the organisms seen in the microscopic field, the vast majority of the bacteria present not showing motility; again, a motile organism when first isolated may show only slight or no motility but later on in bouillon subcultures show active motility. A source of error can be introduced when the bacteria are emulsified in a drop of water which might contain motile bacteria.

For the determination of motility young eighteen-hour-old bouillon cultures are preferable, and the preparation should be made by applying a vaseline ring to the slide, then putting a drop of the bouillon culture in the center of the ring (or a drop of water inoculated from an agar slant growth), then putting on a cover glass. By this method current movement is done away with and the preparations keep for hours. The hanging drop with a concave slide is also used. With this, cut down the light and focus on the margin of the drop with the $\frac{2}{3}$-inch objective before examining with a high dry objective ($\frac{1}{6}$ inch). Do not use the oil immersion objective.

If it were not for the great difficulty of staining flagella, to which the motility of bacteria is due, the characteristics of number and location of flagella might be used in differentiating motile organisms. Organisms with a single flagellum at one pole are designated as monotrichous; with flagella at each pole, amphitrichous and with flagella all around them, peritrichous. The cholera vibrio, a monotrichous organism moves at a rate of 0.03 mm. per second, while the typhoid bacillus, which is peritrichous, has a rate of movement about one-half as great—0.018.

Reaction of Media.—This is a factor of the greatest importance in causing variation in the functions and even morphology of bacteria, and is one which until recently has been almost entirely neglected. In describing an organism at the present time it is always necessary to note the reaction of the media, the temperature at which cultivation took place, and the age of the culture when examined.

While the majority of pathogenic organisms grow within a rather wide range of reaction yet there is usually an optimum reaction at which development best takes place. With other bacteria, as for instance, *B. influenzae* (Pfeiffer) the range is a rather narrow one, so that the matter of reaction is as much to be considered as

that of suitable media. While phenolphthalein titration was an advance over testing with litmus yet the results with such a method were widely divergent when carried out by several bacteriologists under presumably similar conditions. At present the tendency is to adopt a colorimetric method for hydrogen-ion concentration determination—a simple technique is given in the appendix.

The following table is adapted from Dernby:

Name of organism	pH limit	Optimum pH
B. diphtheriae	6.0–8.3	7.3–7.6
B. tuberculosis (horse)	6.0–7.6	6.8–7.2
B. typhosus	6.2–7.6	6.8–7.2
B. coli communis	4.4–7.8	6.0–7.0
B. para "A"	4.5–7.8	6.4–7.0
B. para "B"	4.5–8.0	6.4–7.2
B. suipestifer	5.0–8.2	7.0–7.6
Pyocaneus	5.6.8.0	6.6–7.0
Proteus vulgaris	4.4–8.4	6.0–7.0
Prodigiosus	5.0–8.0	6.0–7.0
Vibrio cholerae	6.4–7.9	7.0–7.4
Cinnabareus	5.0–7.8	6.0–7.0
Pneumococcus I, II, III	7.0–8.3	7.8
Streptococcus, liq	5.5–8.0	6.2–7.0
Staphylococcus	5.6–8.1	7.2–7.6
Gonococcus	6.0–8.3	7.3
B. subtilis	4.5–8.5	6.0–7.5
B. anthracis	6.0–8.5	7.0–7.4
B. anthracoides	6.0–7.8	6.8–7.2
B. influenzae (Pfeiffer)	6.2–7.6	7.0
B. pestis	5.6–7.5	6.5–7.1
B. sporogenes	5.8–8.5	6.0–7.6
B. histolyticus	5.8–8.5	6.0–7.6
B. canadensis	5.8–8.5	6.0–7.6
B. putrificus	5.8–8.5	6.0–7.6
B. perfringens	5.8–8.5	6.0–7.6
B. tetani	5.5–8.3	7.0–7.6
B. botulinus	.....	7.0–7.5
Bacterium tularense	.....	7.3

Gram Staining.—An important method of differentiation is the reaction to Gram's stain. It should be remembered that organisms carried along on artificial media often lose their Gram-staining characteristics; hence it is desirable to determine this staining reaction in cultures freshly isolated.

CHART FOR STUDY OF BACTERIA

Name.....*Source*.....*Date*.....

Form.....*Arrangement*.....

Size, length.....*Breadth*.....*Extreme length*.....

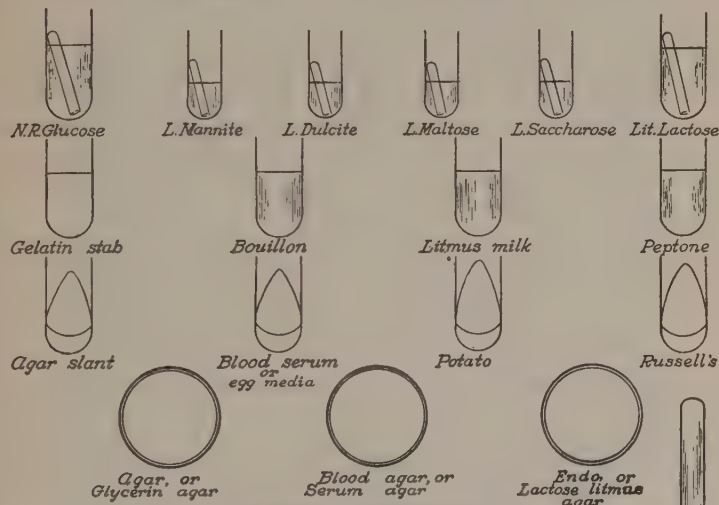
Capsules.....*Spores*,.....*Central*.....*Terminal*.....

Motility.....*Pleomorphism*.....

Staining, reaction, Löffler,.....*Gram*,.....*Acid fast*.....

Pathogenesis:- White mouse.....*Guinea pig*.....*Rabbit*.....

AEROBIC OR FACULTATIVE



OBLIGATE ANAEROBIC

Special media:.....

.....

Notes:.....

.....

.....

(E.R.M.)

FIG. 15.—Bacteriological Chart in use at the U. S. Naval Medical School. The mimeographed sheets are 8 by 14 inches. Red and blue pencil shading characterizes acid or alkaline reactions in sugar tubes. Outlines of colony in plate-rings are made in pencil as is also done on slant figures.

Be sure that the stains, especially the aniline gentian violet and the iodine solution, have not deteriorated. There is no more important stain than this, and none which requires greater experience. The chief causes of conflicting results are (1) working with old cultures and (2) not having satisfactory staining solutions.

Practically all pathogenic cocci are Gram-positive, except the *Gonococcus*, the *Meningococcus*, the *M. catarrhalis*, and the *M. melitensis*.

Practically all pathogenic bacilli are Gram-negative, except the spore-bearing ones, the acid-fast ones, diphtheria and diphtheroid organisms.

The bacillus of glanders is Gram-negative.

Spore Formation.—A very important point in differentiating organisms is that of spore formation. There are numerous nonpathogenic spore-bearing organisms but from a standpoint of human disease we have only to consider one aerobic organism (*B. anthracis*) and the following anaerobic spore-bearers: *B. tetani*, *B. botulinus* and those of infected war wounds which, in the order of their frequency of occurrence, are *B. welchii*, *B. sporogenes*, *B. oedematiens* and *Vibrion septique*. The bacillus of malignant oedema is probably the same as *B. sporogenes*.

Spores are highly refractile, oval to round bodies, surrounded by a thick membrane. When first developing they lie within the bacterial cell but later on this latter degenerates so that we see the spore alone. It must be remembered that certain organisms develop spores only under special environment so that it is necessary to culture organisms, especially on potato or other media suitable for spore development, before considering an organism as a nonspore-bearing one. Temperature requirements are also important; thus *B. anthracis* will not sporulate at temperatures above 43°C. Since the spore-forming organisms resist dessication, heating and application of germicidal agents, the usual method of isolating spore-bearing organisms from non-sporing ones is to heat the mixture to 80°C. for 30 minutes. This kills the nonsporing ones. Spores are best observed in a hanging drop although more beautiful results are obtained by special staining methods. A motile organism, as that of tetanus, becomes immobile previous to sporulation. One method of differentiating spore-bearing bacilli is by the location of the spore in the bacterial cell as when centrally located (clostridium), terminally located (plectridium) or lying between centre and end. Spores develop only in young and actively growing cultures, and the presence of oxygen seems essential for their formation; thus *B. anthracis* never forms spores in the body of an infected animal. It has been stated that some of the pathogenic anaerobes may form spores anaerobically, but Zinsser states that where anaerobiasis is absolute only vegetative bacilli are found. The spores of true spore-forming organisms are endospores. The name arthrospore has been given to certain granules noted especially in old cultures of cocci, but these are not true spores and may be considered as products of degenerative changes.

Liquefaction of Gelatin.—This is a very important means of differentiation. When a room-temperature incubator is not at hand (20° to 22°C.) it is better to put the inoculated gelatin tube in the body-

temperature incubator, and from day to day test for loss of the power of solidifying with ice-water. If the organism digests the gelatin (a liquefier), the medium will remain fluid when placed in ice-water; if the organism is a nonliquefier, the medium in the tube becomes solid. Of course, with this method, we lose the information to be obtained from the shape of the area of liquefaction.

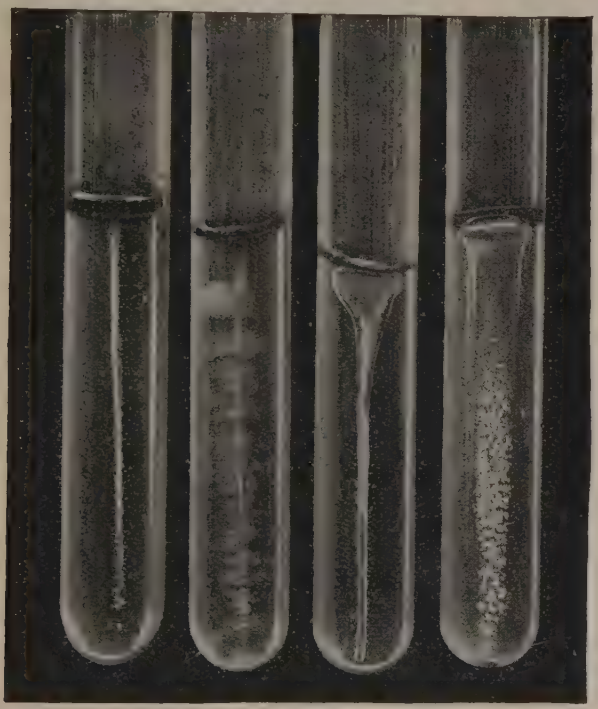


FIG. 16.—Series of stab cultures in gelatin, showing modes of growth of different species of bacteria. (*Abbott.*)

The appearance of the liquefaction area around colonies of liquefying bacteria was formerly regarded as of considerable differentiating value. Before agglutination became a common laboratory procedure the appearance of the liquefied gelatin about a colony of cholera spirilla was always studied but is now rarely thought of. As gelatin is such a clear medium it is very satisfactory in examining colonies with the microscope but this aids little in differentiation. As gelatin is incubated at a low temperature (22°C.), many pathogens will not grow on it and with those which do develop the time for appearance of colonies is twice that for agar plates at 37°C. Gelatin plates are apt to soften and run when handled, hence a source

of danger with such organisms as cholera; especially is this the case where liquefaction also is present. It is in water and milk bacteriology that we need the gelatin plate, since in deciding whether an organism belongs to the proteus or colon group gelatin gives us our best differentiation. Organisms like the proteus group and the cholera spirillum produce an extracellular ferment, gelatinase, which brings about the liquefaction. Bacteria-free filtrates of proteus cultures will liquefy gelatin.

Fermentation.—The effect that certain organisms have on carbohydrates and closely allied non-nitrogenous compounds is of importance in differentiating bacteria. This action is designated "fermentation."

When the bacterial action is on nitrogenous substances, especially protein, the proper term is "putrefaction." If carbohydrates are present in a medium some bacteria will utilize such compounds for energy, sparing the protein. Thus the colon bacillus will not produce indol in media containing sugar. For example, in milk, since it can utilize the lactose, it will produce an acid fermentation and spare the proteins; whereas *B. proteus*, not being able to utilize lactose, attacks the proteins and brings about putrefactive changes.

In practical work we consider fermentation from the standpoints of acid production and gas formation.

Acid fermentation.—For elaborate study we use innumerable carbohydrates such as maltose, saccharose, mannite, dulcite, inulin, etc., but for routine work the only sugar media used are glucose and lactose bouillon. These latter are of the utmost importance in differentiating organisms of the typhoid-colon group, which—following Ford—have primarily been separated by their action on litmus milk—whether turning it pink, or only slightly changing or not changing at all the original color.

Gas production.—In testing for gas production it is best to use the Durham fermentation tube as small amounts of gas may not be easily detected with deep stab cultures into glucose or lactose agar.

If a Durham or Smith tube, or a slant of Russell's double sugar medium, be not at hand the production of gas may be determined by observing bubble formation on the surface of the sugar bouillon culture. As none of the pathogenic cocci produce gas, fermentation tubes are unnecessary where cocci are to be studied. When dealing with cocci and the typhoid-colon group of bacteria, ordinary test tubes containing various sugar bouillons with litmus or Andrade's reagent as indicator suffice for acid-production study. The litmus milk tube gives data as to acid production, coagulation and digestion.

Indol Production.—Certain organisms, especially those of the colon and proteus group, attack tryptophan, one of the amino acids of protein, and produce indol.

Indol is not acted on by its producing organisms and remains in the culture medium, recognizable by the addition of a few drops of H_2SO_4 in the case of cholera (cholera red), or upon addition of a dilute solution of nitrite as well as acid in the case of the colon group. For reliable indol formation the medium should be sugar-free. As a matter of fact indol production seems to be such a variable bacterial function that it has lost much of the differentiating significance it seemed to have a few years ago.

The tests for indol are described on p. 36.

Other Differentiating Measures.—Acid-fast characteristics are very important in separating acid-fast organisms from similar Gram-positive bacilli. Capsule staining is also a valuable means of differentiation when we can be sure that we are dealing with a capsule and not an artefact. Flagellar staining is too difficult to be of much aid in separating bacteria.

Colony Isolation.—Examine the colonies on a Petri plate, at first with the unaided eye, then with a hand magnifying glass or low-power objective, using reflected and transmitted light alternately. Having determined the presence of two or more different kinds of colonies, make a ring with wax pencil around one or more of each kind of colony, numbering them (see Fig. 14). All slides or culture tubes used in determining the species of organism present in the plate should bear the same number as that of the colony from which the material was originally taken.

In picking up material from a colony on a plate I have used a dissecting microscope. With the plate on the stage one can use alternately the light from the substage mirror and that from above (transmitted and reflected), thus giving differentiating information. Having decided on the colony to be selected, touch the colony with a platinum needle while looking through the lens. As the needle is about to touch the colony its point comes into full focus. It is well to support the right hand with the left in order to steady the needle. A convenient procedure is to put a small loopful of water on a clean cover glass and emulsify material from a colony in it. Then invert over a concave slide without vaselining the circumference of the concavity. After examining for motility, smear out and dry the bacterial preparation. Then fix in the flame and stain with aniline gentian violet for two to five minutes. Wash and mount the preparation in water. Afterward, pass through the usual Gram technique.

After this, inoculate the various culture media from similar colonies. *One may inoculate a tube of bouillon from a single colony and later on inoculate the other culture tubes.*

An important point is to wait at least forty-eight hours for colony development (in the case of *B. melitensis*, four to seven days) before reporting on the cultural findings on the agar, blood agar or blood-serum slant or plate upon which the material is smeared (pus, exudate, blood, etc.).

Selective Chemical Inhibitory Agents.—The selective bacteriostatic power of several aniline dyes is often utilized as a means of isolating bacteria which are separated from associated organisms by ordinary methods only with great difficulty and uncertainty. These dyes may be included in the standard formula of a culture medium, as is the case with Petroff, Conradi-Drigalski and Endo, or may be added to a medium at the time it is inoculated as is usually done when making primary cultures of the gonococcus.

It appears that the specific action of dye substances is not necessarily associated with color, but is due to the presence of certain molecular groups which may be colorless. In fact, other compounds, as methenamine, which exhibit neither color nor fluorescence, are similarly used. These substances have been employed clinically also as selective chemotherapeutic agents.

Anaerobiasis.—If it were not for the fact that we have so many facultative anaerobes (organisms capable of growing under anaerobic as well as aerobic conditions) it would be of practical utility to make this biological variation our first step in the study of an unidentified organism. At any rate it is well to remember that the causative organisms of plague, tuberculosis, gonorrhoea, pneumococcal pneumonia and glanders are obligate aerobes while those of tetanus, botulism, gas gangrene and malignant oedema are obligate anaerobes. The pyogenic cocci as well as the causative organisms of cholera, typhoid, paratyphoid and anthrax are facultative anaerobes; they are, however, always studied under aerobic conditions. The colon bacillus as well as organisms of the Friedländer group are also facultative anaerobes.

Partial oxygen tension.—Rosenow has stressed the fact that certain streptococci with localizing tendencies, frequently found in focal infections of dental or tonsillar origin, may fail to grow when cultured either aerobically or anaerobically. They require an intermediate oxygen tension (partial oxygen tension). To obtain such conditions he makes use of a method similar to that of Tarozzi in which pieces of fresh brain, weighing about 1 gram, are dropped into tubes containing a column of broth or 0.5% agar about 4 inches in height. The hydrogen-ion concentration is made from 7.2 to 7.6. The medium is sterilized in the autoclave at 15 pounds for 15 minutes and then is added, from a sterilized concentrated dextrose solution, sufficient sugar to make a 0.2% solution. The medium in the bottom of the tube in the neighborhood of the brain substance is anaerobic and that in the upper part aerobic. These sensitive organisms grow midway in the column but within 24 to 48 hours may work their way up to the surface. Obtaining a primary growth, one may plate out on blood agar to determine haemolytic or green-producing characteristics of such

streptococci, or inject the growth intravenously into medium-sized rabbits to note localization.

Among the nonsporing anaerobes may be mentioned *B. fusiformis* and *M. foetidus*. The pathogenic spirochaetes, *Treponema pallidum*, *Leptospira icterohaemorrhagiae*, *L. icteriodes*, etc., demand anaerobiasis for culturing but are regarded as protozoa. The microscopical globoid bodies of poliomyelitis also require anaerobic conditions in their culture. For methods of culturing anaerobes, see p. 112.

Rockwell's method for growing anaerobes.—When only a few anaerobes are transferred to artificial media they fail to grow if the oxygen and respiratory CO_2 are absorbed by pyrogallic acid and caustic alkali, but will grow upon removal of oxygen provided some CO_2 is present. In order to insure the presence of CO_2 in the culture tube the following solution should be used in place of caustic alkali:

Sodium bicarbonate	50.00 Gms.
Acid sodium phosphate	0.75 Gms.
Water	500.00 cc.

Keep tightly corked.

This method is particularly useful in growing parasitic streptococci. Cultures are made on dextrose ascites agar; the cotton plug is shortened by clipping with scissors and the plug pushed into the test tube to the upper level of the medium; a tight wad of absorbent cotton is placed on top of this; and then is added 0.5 Gm. pyrogallic acid and 1 cc. of the charged alkali solution and the test tube is corked tightly with rubber stopper.

Often when the material contains several bacteria the growth obtained in this way will, in 24 hours, show a pure culture of streptococci.

Koch's Postulates.—Should an organism be encountered in an original investigation, the following four requirements should be fulfilled before an etiological relationship can be regarded as established.

1. The organism should be constantly present in that particular pathological condition. 2. It should be isolated in pure culture from the pathological material. 3. When inoculated in pure culture into suitable animals it should reproduce the pathological conditions. 4. It should be recovered in pure culture from the experimental animal. For various reasons, such as lack of suitable animals or proper artificial media, these requirements are impossible of execution with several organisms which are, however, generally recognized as the specific causes of certain diseases.

Animal Experimentation

The experimental animals most frequently employed in the diagnosis of bacterial diseases are the guinea pig, the rabbit, the white rat and the white mouse. In the following diseases the most suitable animals for inoculation are:

1. Tetanus—mice or guinea pigs, subcutaneously. The spasms begin in the limbs nearest the site of inoculation.

2. Pneumococci and streptococci—mice subcutaneously at root of tail or intraperitoneally; or rabbits, intravenously.

3. Staphylococci—rabbits.

4. Diphtheria, tuberculosis, anthrax and botulism—the guinea pig subcutaneously.

5. Glanders and cholera—the guinea pig, intraperitoneally.

6. Plague—guinea pigs, cutaneously or subcutaneously.

7. Gas bacillus—rabbits intravenously, killing them in 5 minutes and incubating dead animal about 7 hours.

8. Rabies—rabbits, subdurally, with salt solution emulsion of medulla of suspected dog.

In the cutaneous method of infection the material, as from a plague bubo, or the sputum from pneumonic plague, is thoroughly rubbed with a glass rod upon the shaven surface of the guinea pig.

In the subcutaneous method one can use a hypodermic needle (the all-glass syringe with platino-iridium needle is the best) or an opening can be cut with scissors, a pocket then opened up with forceps and a piece of tissue inserted to the bottom of the pocket with the forceps. The incision may be sealed with collodion.

The marginal ear vein of the rabbit is used for intravenous inoculation. This can be made to stand out by the application of either hot water or xylol.

In intraperitoneal injections the animal is best held head down so that the intestines gravitate headward. The shaven skin is pinched up and the needle inserted in the median line. The gut is less liable to injury if the needle be passed obliquely through the skin, then vertically through muscles.

In certain cases infection is brought about by feeding the material to the experimental animal. The material may be mixed with the food or introduced into the stomach by a tube. In the investigation of respiratory tract infections the material is introduced into the upper respiratory tract of the animals by spraying or otherwise. In experimentation as to food deficiencies the white rat is used to demonstrate lack of fat soluble A; the pigeon, lack of water soluble B, and the guinea pig to show deficiency in the antiscorbutic vitamin (water soluble C).

Culturing Material Obtained at Biopsy or Necropsy of Man or Animals

It is customary to sear with a heated knife or spatula a spot on the surface of the organ from which cultures are to be made. Then introduce at this point a sterile platinum spud, with which tubes of culture media are inoculated. The platinum loop may be used where an incision has been made into the organ with a sterile knife.

A bacteriological capillary pipette is a good instrument for taking up material. After some practice one can do good work with a rubber bulb capillary pipette, especially in taking up blood from right heart or blood vessels. When great pre-

caution is necessary to insure sterilization of the surface, as in culturing a removed gland or organ, the piece of tissue may be dropped into 5% formalin solution for a few minutes, washed in sterile salt solution, next placed in a sterile Petri dish and the material obtained from the center; or it may be dropped for a few seconds into boiling

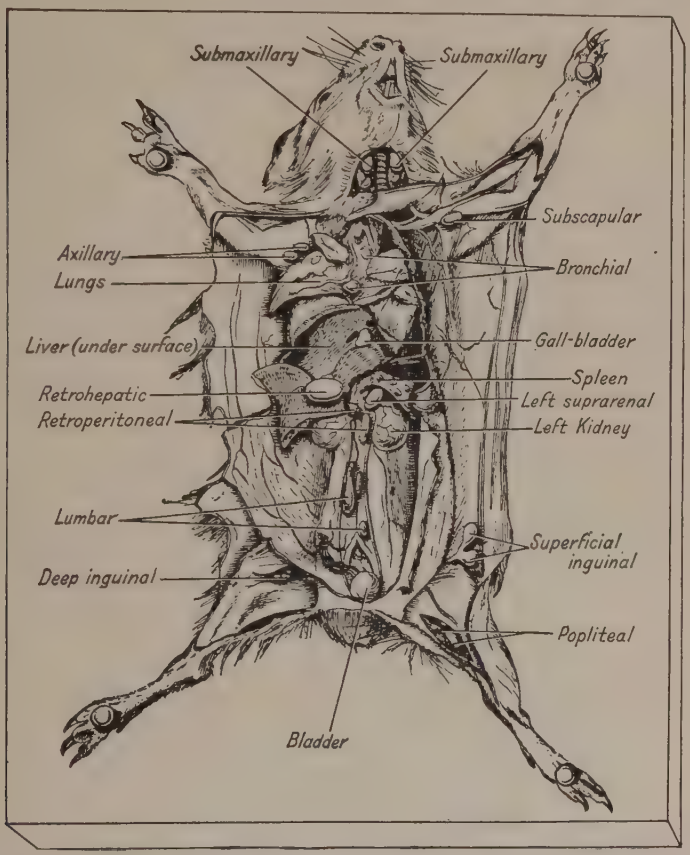


FIG. 17.—Cadaver of guinea pig, showing important organs. (*From Eyre's "Bacteriological Technique."*)

water. Before performing a necropsy on experimental animals it is well to dip the dead animal into 3% trikresol solution.

In autopsying small laboratory animals they may be tacked down by each foot to a wooden board, the whole being thrown in the furnace after the autopsy. When pans are used these should be put in the autoclave.

CHAPTER V

STUDY AND IDENTIFICATION OF BACTERIA—COCCI. KEY AND NOTES

Streptococcus Forms.—Cells in short or long chains, never in packets.

I. Parasitic or semiparasitic.

A. Haemolytic group.

(a) Produce wide zone of haemolysis around colonies on blood agar.

Type β (beta) of Smith and Brown. In this group belong the following types:

1. Streptococcus pyogenes (*S. erysipelatis, S. puerperalis*).
2. Streptococcus scarlatinae (*S. anginosus*).
3. Streptococcus mixtos (*S. infrequens, S. haemolyticus II* Holman).
4. Streptococcus equi.
5. Streptococcus mastitidis.
6. Streptococcus cuniculi.
7. Streptococcus felini.

(b) Produce narrow zone of haemolysis around colonies on blood agar.

8. Streptococcus stenos.

B. Viridans group. Produce methaemoglobin, no definite haemolysis. Colonies surrounded by green or brown colorations. Type α (alpha) of Smith and Brown. In this group belong the following types:

1. Streptococcus mitior (*S. viridans, S. mitis, S. salivarius*).
2. Streptococcus fecalis.
3. Streptococcus equinus.
4. Streptococcus bovis.
5. Streptococcus ignavus.

C. Non-haemolytic group.

Type γ (gamma) of Smith and Brown. Produce no change on blood agar.

1. Streptococcus anhemolyticus.
2. Streptococcus saprophyticus.

II. Saprophytes occurring chiefly in dairy products and found principally in sour milk and in the ripening process of several varieties of cheese. In this group belong the lactic acid bacteria and include S. cremoris, S. thermophilus, S. lactis, S. kefir, and several other nonpathogenic ones.

Differentiation of Streptococci on basis of haemolysis, carbohydrate fermentation and absorption of agglutinins.

	Haemolysis	Lactose	Mannitol	Salicin	Milk	Serological type
S. pyogenes	+	+	−	+	A	I
S. scarlatinae*	+	+	−	−	A	II
S. mixtos*	+	+	+	+	A	III
S. equi	+	−	−	+	A	IV
S. mastitidis	+	+	−	+	AC	V
S. cuniculi	+	+	−	+	AC	VI
S. felini	+	+	−	+	A	VII
S. stenos*	+	+	+	+	A	VIII
S. mitior	−	+	−	+	AC	
S. fecalis	−	+	+	+	AC	
S. equinus	−	−	−	+	AC	
S. ignavus	−	+	+	+	O	
S. anhemolyticus	−	−	−	+	O	
S. saprophyticus	−	+	−	+	AC	

+ = Acid.
− = No change.
A = Acid.
C = Coagulation.
O = No change.

* Certain species of these organisms give different fermentation reactions although they correspond to same serological types.

Note.—In the above differentiation one should use the blood agar recommended on page 43. The plates should be placed in the incubator, as alpha type will show an outer haemolyzed ring if kept in the cold. The deep colonies are of more value in differentiation than the surface ones. For fermentation reactions it is necessary that the various sugars be added to media in which the streptococci grow luxuriantly. Some use Hiss' serum water (See p. 37).

Differentiation of streptococci on the basis of their ability to ferment carbohydrates gives no concordant results with serological tests in the form of agglutination reactions and absorption of agglutinins. Species of streptococci are best grouped on the basis of their action on blood agar and serological reactions and, secondarily, on the basis of carbohydrate fermentation.

Staphylococcus Forms.—Cells as a rule in irregular groups, rarely in packets. Pigment white or orange, or less commonly lemon-yellow.

I. Orange pigment. Lactose fermented. Gelatin liquefied.
   (1) Staphylococcus aureus.

II. Lemon-yellow pigment.
   (2) Staphylococcus citreus.

III. White or colorless growth on solid media.
   (a) Ferment lactose, sucrose, liquefy gelatin.
      (3) Staphylococcus epidermidis.
   (b) Ferment lactose, sucrose, mannitol.
      (4) Staphylococcus albus.
   (c) Ferment lactose, sucrose, mannitol, raffinose.
      (5) Staphylococcus pharyngis.

Tetragenus Forms.—Cells in pairs and irregular masses. Ferment glucose and lactose.

(1) Gaffkya tetragena. (*M. tetragenus*).

Sarcina Forms.—Division occurs in three planes, producing regular packets. Growth on agar abundant, usually with the formation of yellow or orange pigment.

I. Nonmotile forms.
  A. Yellow pigment formed.
    (a) Gelatin not liquefied.
      (1) Sarcina ventriculi (Stomach contents of man and animals).
      (2) Sarcina conjunctivae (conjunctiva).
    (b) Gelatin liquefied.
      (3) Sarcina flava (air).
      (4) Sarcina lutea (air, soil, water).
  B. Orange pigment formed.
      (5) Sarcina lactea (fresh milk).
      (6) Sarcina aurantiaca (air, water).
  C. No pigment formed (Gelatin not liquefied).
      (7) Sarcina hamaguchiae (soy bean mash).
II. Motile forms.
  A. Yellow pigment formed. Gelatin not liquefied.
      (8) Sarcina citrea (air).
      (9) Sarcina ureae (urine).

Diplococcus Forms.—Cells usually in pairs. Grow poorly on artificial media. Oval to lancet-shaped forms, less frequently in chains. Bluish to greenish tint on blood agar without haemolysis. Bile soluble.

(1) Diplococcus pneumoniae (*Pneumococcus*). Four principal types recognized on basis of agglutination (See text).

Micrococcus Forms.—Cells in plates or irregular masses (never in chains or packets). Facultative parasites. Found in oral cavity and urine. Aerobic and anaerobic species. Nonpathogenic for man.

Gram Negative Cocci. Neisseria.—Cells normally in pairs.

A. Grow only at 37°C. on special culture media containing blood, blood serum, starch or on plain agar with vitamin.
  (1) Neisseria gonorrhoeae (*Gonococcus*).
  (2) Neisseria intracellularis (*Meningococcus*). Four distinct serological types of meningococcus, differentiated on basis of agglutination.
B. Grow well at 22°C. on ordinary culture media.
  (a) Non-chromogenic.
    (3) Neisseria catarrhalis.
    (4) Neisseria sicca.
  (b) Chromogenic.
    (5) Neisseria perflava.
    (6) Neisseria flava.
    (7) Neisseria subflava.

See page 100 for carbohydrate fermentation reactions.

Streptococcus Forms

Those cocci tending to arrange themselves in chains are usually described as streptococci. (Ogston, 1881; Rosenbach, 1884.)

It is often difficult to distinguish streptobacilli from streptococci morphologically and the same is true of diplococci and diplobacilli. These bacillary pairs and chains, however, often show bipolar staining and are almost invariably Gram-negative with the exception of the small lactic acid-producing bacilli of milk.

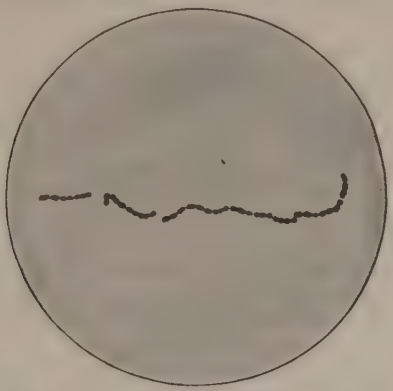


Fig. 18.—*Streptococcus pyogenes.* (*Kolle and Wassermann.*)

While streptococci tend to assume chain formation in pus and tissues they often appear as diplococci in blood. The enterococcus and pneumococcus may form chains in fluid cultures but in material from the body fail to show chain formation.

Streptococci as normal findings.—The essential point to bear in mind is that the finding of a streptococcus does not necessarily explain an infection, because normally streptococci are among the organisms most frequently and abundantly found in plates made from normal buccal and nasal secretions. It is well to be very conservative when reporting streptococci as the etiological factor from lesions of the throat or nose.

Probably the most practical point in the differentiation of streptococci, next to that of pathogenicity, is the occurrence of long or short chains; the virulent ones tending to appear in chains of from 10 to 20 cocci, while the normal inhabitants of the nose, mouth and faeces generally tend to be in shorter chains.

Most bacteriologists now regard haemolysis, absorption of agglutinins, and action on carbohydrates as of great practical value in differentiation. Such means have been employed in the preceding table of streptococcus forms. Work by Dochez and others would indicate, that there are a number of strains of *S.*

haemolyticus, as determined by agglutination and protection tests. It is generally accepted that *S. viridans* is a name which has been attached to many distinct streptococcal strains.

It must be remembered that there are various streptococcal organisms associated normally and pathogenically with various animals. Of these we may note the streptococcus of strangles in the horse and that of contagious mammitis of cows. These latter do not appear to be causative of streptococcus sore throat in man, which is due to a human strain which is capable of invading the udder and infecting the milk.

It has been a mooted question as to whether *S. lacticus* represents a streptococcal organism or belongs to a group of small, Gram-positive, chain-forming bacilli.

Avery and Cullen have shown that human strains of haemolytic streptococci cease to grow when the hydrogen-ion concentration of the medium reaches pH 5, while the strains of bovine origin grow in a more acid medium (pH 4.3).

Cultural characteristics.—Human streptococci are facultative aerobic organisms and grow best at body temperature. In bouillon the long-chain streptococci tend to form a flocculent growth which adheres to the sides of the test tube and after three or four days falls to the bottom as a granular precipitate. With the short-chain streptococci there is more of a tendency to a turbidity.

Growth on whole blood media is most satisfactory for diagnostic as well as other purposes. The cocci can be streaked on the surface of blood-agar (cooled to 42°C.) and the content mixed and poured into a sterile Petri dish. Rosenow's glucose-brain-broth is most satisfactory for blood culture work. Stock cultures are best kept on chocolate agar (defibrinated blood agar heated to 75°C.), transplanting once a month. Due to spontaneous or self-agglutination of many strains there is often interference with agglutination and it is necessary to make transplants daily in Rosenow's broth until uniform suspensions are obtained.

(See key and notes for carbohydrates and blood agar plate differentiation, p. 86.)

Differentiation of streptococci from pneumococci.—The most important criterion is the solubility of pneumococci in bile. To carry out this test add to 2 cc. of a 24-hour bouillon culture of the organism about 0.1 cc. of ox or other bile which has been filtered and sterilized, or a 10% solution of sodium taurocholate. The mixture should remain in the incubator for ½ hour. Streptococcus cultures remain unchanged while the turbidity of the pneumococcus culture clears up.

Another test which is of value is that of inulin fermentation. The inulin solution in water is autoclaved to destroy sporulating organisms and is then added to Hiss' serum water, in the proportion of 1 to 100, with litmus indicator. Pneumococci ferment inulin with acid production within 48 hours while streptococci have no such effect.

Formerly we recognized organisms having marked capsule formation and mucilaginous growth as *S. mucosus*. As a rule these organisms are pneumococci belonging to group *III* (*Pneumococcus mucosus*), but we do recognize a *S. mucosus* which is not bile-soluble, does not ferment inulin, is not so pathogenic for mice and has rather a tendency to produce slight haemolysis in blood agar.

Virulence.—As regards virulence, this is exceedingly variable—it is soon lost, but may be restored either by inoculating streptococci

along with various other organisms or by passage through successive rabbits.

The rabbit is the most susceptible animal. Inoculation should be made in one of the prominent ear veins. If the needle of the syringe is not inserted in the vein, it will be shown by difficulty in forcing in the material and a swelling will immediately appear.

Recently isolated cultures from human infections are not very virulent for animals. Passage through the rabbit, however, enormously increases the virulence. Mice are more conveniently inoculated at the root of the tail. If the culture is very virulent, the infection becomes generalized and death occurs in two or three days. If less virulent, a local abscess forms.

Almost without exception, human streptococci are Gram-positive. Their colonies are quite small but distinct and discrete. In appearance the colonies of streptococci and pneumococci are practically identical. In a blood-serum throat culture *Pneumococcus* and *Streptococcus* colonies are the smallest, diphtheria ones are quite small and discrete, but slightly flatter. (Always examine the water of condensation for streptococci.) The *Sarcina* and *Staphylococcus* colonies are much larger.

Streptococcic colonies on blood agar are much more moist and luxuriant than on ordinary agar. A very important point, in judging whether a *Streptococcus* or other organism is pathogenic in a given infection, is to examine smears from the pus or other material in a Gram-stained specimen for information as to abundance and, in particular, phagocytosis of any organism, before plating out.

Pathogenic effect.—Streptococci are commonly the cause of diffuse phlegmonous inflammations, while the staphylococci cause circumscribed lesions. Streptococci cause necrosis and do not characteristically produce pus. The importance of the *Streptococcus* as a secondary infection in diphtheria, tuberculosis, smallpox, scarlet fever, and even in typhoid fever must always be kept in mind. It is this infection which does not respond to diphtheria antitoxin, and not the diphtheria one.

Streptococcus viridans.—Many of the green-colony streptococci to which this name has been given are nonpathogenic and those associated with disease production cause subacute conditions rather than the fulminating manifestations of the haemolytic group. The viridans group are the common organisms found in tooth abscesses as well as in tonsil and sinus focal infections. These focal infections have a frequent relation to joint and heart conditions. Various workers have claimed the isolation of green streptococci from joint fluids but in the hands of most workers such results have not been obtained. In the protracted course of ulcerative (malignant) endocarditis the isolation of a viridans organism from the blood has been common. Such cases frequently give embolic lesions. Chorea and the green streptococcus have been considered as connected.

Rosenow has reported on the rather constant presence of streptococci in gastric and duodenal ulcers removed at operation, under which circumstance the number and variety of bacteria present are comparatively few. The strains from 27 chronic

ulcers gave grayish-green colonies on blood plate, were in short chains and pairs, produced much acid and turbidity in dextrose broth and showed a low-grade virulence. When injected into dogs, rabbits and guinea pigs they showed a tendency to localize in the mucosa of stomach and duodenum, causing ulceration in a large percentage of cases. Rosenow believes poliomyelitis a streptococcal infection. Under anaerobic or partial oxygen tension conditions, streptococci may appear as exceedingly small bodies, more or less resembling the filterable virus globoid bodies reported by Flexner and Noguchi as the cause of poliomyelitis.

Streptococci as well as colon bacilli are always to be thought of in connection with cholecystitis and appendicitis.

Scarlet fever streptococci.—Klein in 1886 isolated streptococci from scarlatina throats and called them *S. scarlatinae.* Evidence in favor of this organism being the cause of scarlet fever has been produced in recent years by Dochez and the Dicks. In selected volunteers the Dicks succeeded in producing experimental scarlet fever by swabbing the throats with haemolytic streptococci obtained from a case of scarlet fever. Nicolle confirmed this observation in 1926. In addition the Dicks were able to show that the *S. scarlatinae* produces an exotoxin which can be demonstrated in Berkfeld filtrates, which, when injected intradermally into susceptible individuals produces a characteristic skin reaction. This toxin requires high temperatures for prolonged periods to destroy its activity.

The Dick Test.—This reaction is used to determine susceptibility or immunity to scarlet fever. The test is carried out in the same manner as the well-known Schick test for diphtheria. Skin reactions appear in 4 to 12 hours after injection and readings are made after 22 hours and not later than 24 hours. At the height of the positive reaction there is a circumscribed area of redness and infiltration varying in size from 1.0 to 3 or 4 cm. according to the susceptibility of the individual. A reaction which has entirely faded in 24 hours is negative. It has been found by the Dicks, Dyer and others that the percentage of children giving pseudoreactions to the Dick toxin is small. For this reason it is customary to use no control.

Immunization against scarlet fever.—Fraser and Graham have shown that a Dick-positive individual may be rendered Dick negative (passively immunized) in 24 to 48 hours by the administration of from 1 to 5 cc. of scarlet fever antitoxin and that this prophylactic dose confers protection for from 2 to 4 weeks. Active immunization against scarlet fever is carried out by giving a course of four to five subcutaneous or intramuscular injections of increasing dosages of toxin expressed in skin test doses. Three weeks following the last injection it is essential that another Dick test be made to determine whether or not immunity has developed. Larson has been able to immunize against the disease by giving one injection of an antigen made from killed scarlet fever streptococci to which has been added castor oil soap (ricinoleated antigen). The important question as to the most suitable doses

of toxin to administer, the total amount of toxin which should be given and the question of duration of immunity which results, remain as yet unanswered.

Scarlet fever antitoxin.—Scarlet fever antitoxin is being produced by several methods. For several years convalescent serum obtained from patients who had recently recovered from scarlet fever has been used with success in the treatment of certain cases of scarlet fever. Moser found that the serum of horses heated with broth cultures of scarlatinal streptococci was valuable in the treatment of scarlet fever. Dochez and later the Dicks conclusively established the fact using *S. scarlatinae.* The dosage of the serum varies from 200,000 to 500,000 neutralizing (so-called skin test) doses. The convalescent or antitoxic serum may be used as a diagnostic test, the Schultz-Charlton phenomenon. A small quantity (0.2 to 5 cc.) of the antitoxin is injected into the skin of the patient at an erythematous point and a definite area of blanching is produced at the site of injection after five or six hours. The serum neutralizes the toxin in the tissues and causes the disappearance of the rash in scarlet fever.

Measles.—Tunnicliff, Ferry and Fisher and others have published results of investigations which lead them to believe that a variety or strain of streptococcus is the causative agent of measles. The microorganism described by Ferry and Fisher is a small Gram-positive aerobic green producing streptococcus appearing in pairs and chains, which produces a soluble toxin which they claim is specific in measles. Tunnicliff using a suspension of green streptococcus as antigen in skin tests claims to be able to differentiate between persons susceptible to measles and those who were immune. Ferry and Fisher claim that the toxin elaborated by *S. morbilli* may be used in a skin test for susceptibility or immunity, and as an actively immunizing agent in man or lower animals. Tunnicliff's measles immune goat serum has been used in the prophylaxis of measles, those receiving the serum within five days after exposure being protected in about 90 per cent. of the cases for 2 to 3 weeks, others passing through an attenuated form of the disease.

Degkwitz has shown that 3.5 cc. of convalescent measles serum will prevent the development of measles in an exposed child. Using cultures from filtrates of nose and throat secretions or blood from cases of measles taken about the time of the appearance of the rash, he states that he has produced experimental measles in monkeys and that the serum from sheep treated with these cultures may be used to prevent the development of measles in exposed children.

Erysipelas.—Birkhaug states that by means of agglutination and absorption test he has demonstrated 91 per cent. of haemolytic streptococci isolated from cases of erysipelas to fall into a definite group, which was serologically distinct from the scarlet fever group and also from streptococci isolated from common septic conditions. A serum prepared from the erysipelas strains protected rabbits against infection with similar strains but did not confer immunity against streptococci from other sources. The intracutaneous injections of these erysipelas organisms into normal rabbits produced a condition strikingly resembling human erysipelas, and with a toxin obtained from the organisms a skin reaction akin to the Dick reaction could be demonstrated. He further reports results of the use of an antiserum prepared from the erysipelas streptococci and used in human erysipelas. The clinical trial of such a serum in 60 patients suggested that it had striking therapeutic effects when administered early in the disease and in adequate dosage. The average

dose given was 100 cc. of the unconcentrated serum and in most cases a single dose sufficed. The injection was usually followed by a critical drop in temperature, an amelioration of the general condition, and the disappearance of the eruption. In late cases the chief effect of the serum was in the amelioration of the toxic condition, its local results being less striking.

It has been claimed that acute articular rheumatism is due to a short-chain streptococcus (*M. rheumaticus*), which is best isolated from material from an acute joint infection, but may also be isolated occasionally from the blood. It produces much acid and clots milk in two days. The growth is described as being more luxuriant than that of *S. pyogenes*. It is about 0.5μ in diameter.

Sarcina Forms

These are best observed in hanging-drop preparations, when they can be seen as little cubes, like a parcel tied with a string; by noting them when turning over, it will be seen that they are different from the tetrads which divide in only two dimensions of space. At times the packet formation is not perfect and it will be difficult to distinguish such as sarcinae. All sarcinae stain by Gram. If the staining of sarcinae be too deep it may obscure the lines of cleavage.

Various sarcinae have been isolated from the stomach, especially when there is stagnation of stomach contents. Sarcinae have also been found in the intestines. In plates the *S. lutea* is frequently a contaminating organism, being rather constantly present in the air. The demonstration of sarcina morphology should always be made from liquid media, as bouillon. Urine makes an excellent medium.

Gram-positive Cocci

It has been found convenient to divide all cocci which do not show chain or packet formation into two classes, namely, those which are gram positive and those which are gram negative.

M. tetragenus. (*Gaffkya tetragena.*) Gaffky, 1881.—This organism is frequently found associated with other organisms in sputum, especially with tubercle and Pfeiffer bacilli. Smears from sputum or pus show a large coccus arranged in fours and surrounded by a broad capsule. In cultures the capsule is often absent. The colonies, which are rather slow-growing, are white, slightly smaller than those of staphylococci and are quite viscid.

It does not liquefy gelatin but produces acid in glucose, lactose, saccharose and mannite. Milk is slightly acidified, and is usually coagulated in 1 to 3 days but the coagulum is not digested. It was formerly considered unimportant in disease, but the idea now prevails that it is responsible for many abscesses about the mouth,

especially in connection with the teeth. Injected subcutaneously into Japanese mice, it produces a septicaemia and death in three or four days. The blood shows great numbers of encapsulated tetrads. It has been reported twice as a cause of septicaemia in man.

Staphylococci. (Ogston, 1881).—To cocci dividing irregularly and usually forming masses which are likened to clusters of grapes the term *Staphylococcus* is applied. While there have been experiments which show that by selecting pale portions of a yellow colony, eventually a white colony could be produced, yet, as a practical consideration, it is convenient to consider at least two types of staphylococci, the *Staphylococcus aureus* and the *Staphylococcus albus*. In culturing from the pus of an abscess or furuncle we generally obtain a golden coccus, while with material from the nose or mouth, the *Staphylococcus* colonies are almost invariably white. As regards the common skin coccus, this will be found to produce a white colony. A coccus which very slowly liquefies gelatin and has been supposed to cause stitch abscesses is the *S. epidermidis*.

While it is customary to look for a golden colony in the case of organisms showing virulence, yet at times a cream-white colony may develop from cocci of great virulence. Staphylococci show marked resistance to dessication and dried pus may contain live organisms for months. Old bouillon cultures of staphylococci contain a ferment-like substance, leucocidin, which disintegrates leukocytes. Leucocidin is a soluble toxin produced by the staphylococcus and is like other soluble toxins. We have an antileucocidin which may be normally present in human serum. Old bouillon cultures may show also an haemolysin, and when filtered and injected into animals show destructive action on cells of various organs. Nonvirulent cultures do not seem to produce haemolysins. Amyloid change may be caused in animals by repeated injections of either living or dead cultures.

The *S. citreus* is considered as of very feeble pathogenic power. The *Staphylococcus aureus* grows readily at room temperature, but better at 37°C. It coagulates milk and renders bouillon uniformly turbid. It grows on all media, as blood serum, agar, potato, etc. It has been proposed to distinguish it from skin staphylococci by its power of producing acid in mannite. Acid is also produced in glucose, lactose and saccharose media. Russell's double sugar with Andrade's indicator is an excellent medium for demonstrating pigment and sugar reaction. Ordinarily the individual cocci are about 0.8μ in diameter, but they vary greatly in size according to the age of the culture and other conditions.

Pathogenicity.—The "aureus," as it is frequently called, is not only often found in circumscribed processes, such as boils, carbuncles and at times in acne pustules, but it is a frequent cause of pyaemia, osteomyelitis, endocarditis, etc. In the tropics staphylococcal infections often show great virulence and clinically may resemble streptococcal ones. The organisms in smears from such erysipelatoid lesions show diplococcal morphology and are often phagocytized. A pemphigoid eruption in

children is often staphylococcal (pyosis). Pyelitis may be due to staphylococci as is also true of pyelonephritis. In staphylococcal sepsis multiple abscesses in various organs are common. Blood cultures are more apt to be negative than with a streptococcal sepsis.

In infection of bone tissue the *Staphylococcus* is by far the most frequent cause (osteomyelitis, periostitis). It is well to remember that insignificant staphylococcal infection may lead to septicaemia. Such infections are quite common in diabetes and other disturbances of metabolism. In the tropics, where resistance is often lowered and staphylococcal skin infections common, continued fevers are often septicaemias. It is the organism most frequently concerned in terminal infections, the lowered resistance of the patient permitting its passage through barriers ordinarily resistant. Not only should this be kept in mind when such organisms are isolated at an autopsy, but as well the fact that their entrance may have been agonal or subsequent to death.

FIG. 19.—Gelatin culture. *Staphylococcus aureus* one week old. (*Mac Neal.*)

Immunity.—Injections of cultures of staphylococci increase especially the opsonins. In broth-culture injections, antileucocidin is also found in the serum of the immunized man or animal. Agglutinins are also formed which seem to act alike on various strains, whether "aureus" or "albus," but virulent strain agglutinins do not seem to clump nonvirulent strains. Vaccines have been most successful in the treatment of staphylococcal infections. They stimulate phagocytosis. Serum treatment has not proven of value.

Resistance.—Of all nonsporing bacterial organisms staphylococci are the most resistant to dessication, heating and germicides. When contained in dried material destruction of staphylococci is particularly difficult, requiring temperatures approximating 80°C. for almost an hour to kill them. Even 100°C., if of only a few minutes application, may fail to sterilize.

The Pneumococcus of Fraenkel. (*Diplococcus pneumoniae.*) Pasteur and Sternberg in 1880. Fraenkel, 1884, isolated it from normal persons as well as pneumonia patients, and inoculated mice and rabbits; hence, Fraenkel's organism. Weichselbaum accurately differentiated organisms causing pneumonia in 1886.—This is by far the most common cause of lobar pneumonia. It is also frequently found in meningitis, empyema, endocarditis and otitis media. It should not be confused with the pneumobacillus of Friedländer, which, although possessing a capsule like the *Pneumococcus*, differs from it by being Gram-negative, being a bacillus and having large viscid colonies. The

Pneumococcus is the cause of more than 80% of the cases of lobar pneumonia.

In 529 cases the Rockefeller Institute reports give the following causation table:

Diplococcus pneumoniae (*Pneumococcus*)	454
Friedländer's bacillus	3
Bacillus influenzae (*Pfeiffer*)	6
Streptococcus pyogenes	7
Streptococcus mucosus	1
Staphylococcus aureus	3
Cases of mixed infection with combinations of *Staphylococcus aureus*, Friedländer's bacillus, *B. influenzae*, *Streptococcus pyogenes*, and *Streptococcus viridans*	6
Undetermined (most of them occurring before accurate methods for determining the etiologic agent had been devised)	49

Morphology.—The *Pneumococcus* in pathological material and in cultures in albuminous media shows as two lanceolate bodies with bases apposed (less frequently oval and rarely round) set in a capsule. Even when the short-chain arrangement is

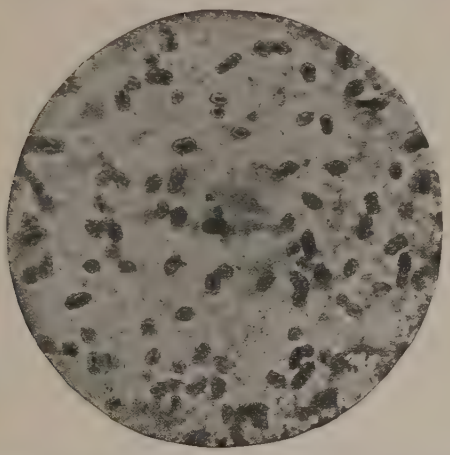


FIG. 20.—*Pneumococcus*, showing capsule, from pleuritic fluid of infected rabbit stained by second method of Hiss. (*Mac Neal.*)

present these are made up of diplococci. In agar or broth cultures, it does not show a capsule.

Cultural characteristics.—It does not grow below 20°C. and is best cultivated on blood serum, or blood agar. On plain agar it grows as a very small dew-drop-like colony, which is slightly grayish by reflected light. Colonies on blood agar plates show a zone of greenish coloration around the colony. It produces considerable acid, thus acidifying and usually coagulating litmus milk. It produces acid in inulin media, which the *Streptococcus* fails to do. The most important differentiating point

is its solubility in bile. The colony is smaller and more transparent than *Streptococcus* colony. The best medium for differentiating is the serum of a young rabbit; in this it grows as a diplococcus, while streptococci show chains. Rosenow, by combining passage in animals with culturing symbiotically with *B. subtilis*, claims to have changed the *Pneumococcus* into a haemolytic *Streptococcus*. The best method of isolating it in pure culture is to inject the sputum into the marginal ear vein of a rabbit or subcutaneously into a mouse. Death results from septicaemia in about two days and the blood teems with pneumococci. In typing pneumococci from sputum we inject the well washed sputum into the peritoneal cavity of the mouse. Usually the *Pneumococcus* quickly loses its virulence, and also dies out in a few days unless transferred to fresh media. Virulence is quite variable; it is attenuated by subculturing and exalted by animal passage. Usually the more toxic a case of pneumonia the more virulent the pneumococci, so that 0.000001 cc. of a broth culture may kill a mouse in thirty-six hours. The best medium for its preservation is rabbit's blood agar; this also maintains the virulence. On this medium the colonies are larger than on agar and they present a greenish appearance. They are flat, ringed and surrounded by a greenish zone of methaemoglobin.

The *Pneumococcus* growth emulsifies very readily and evenly so that suspensions for vaccines are easily made.

Pneumococcus as a normal finding.—It is a well known fact the *Pneumococcus* is a frequent inhabitant of the nasal, pharyngeal, and buccal cavities of persons not suffering from disease. The explanation of infection is either on the ground of lowered resistance of the patient or enhanced virulence of the organism. Vaccines appear to be valueless in pneumonia but may be useful in local infections.

The blood and sputum in pneumonia.—In a study of blood and sputum cultures from 32 cases of lobar pneumonia Hastings and Boehm found blood and sputum positive bacteriologically in 11 cases. In 9 of these cases the *Pneumococcus* was isolated and in 2 a haemolyzing *Streptococcus*. In the other 21 cases the sputum cultures were bacteriologically positive in 18 of the cases and negative in 3. In 9 cases the *Pneumococcus* was isolated, in 2 cases *B. coli*, in 1 case *M. catarrhalis*, in 1 case a *Staphylococcus*, in 2 cases staphylococci and streptococci, in 1 case *B. influenzae* (Pfeiffer). The percentage of positive blood cultures was 30.3. Cole obtained 30% of positive blood cultures. The blood was taken into flasks of bouillon in dilution of 1–50. In the cases giving positive blood cultures the mortality was 55% in the negative ones 8%.

Types of pneumococci.—Neufeld showed that different strains of pneumococci differed immunologically. Cole and his colleagues at the Rockefeller Institute Hospital have elaborated his work and divided pneumococci into four groups. Of these, organisms belonging to group III can be recognized in a Gram-stained preparation by their large capsules and in cultures by their viscid colonies, the groups I and II can be differentiated by serum reactions and to group IV belong pneumococci which cannot be placed in the other groups. The common name for group III pneumococci is *P. mucosus*.

In cases of pneumonia group I was found in 33%, group II in 31%, group III in 12% and group IV in 20%. The mortality in such cases was 23, 32, 45 and 16% respectively.

Epidemic strains.—Types I and II are the strains most often present in epidemics of pneumonia and have been referred to as "epidemic strains." They are rarely found in the throats of those who have not been in contact with cases, the normal throat types usually belonging to Group IV.

In a study of the saliva of 297 noncontacts, group I organisms were only found once and group II ones not at all. Contacts with type I cases gave 13% and with type II 12% of positives. These contact carriers usually harbored the organisms for only three or four weeks. Even cases of the disease usually cease to show these epidemic types in their sputum within 3 or 4 weeks. For methods of differentiating the groups see p. 245 and p. 247.

Protection tests are now regarded as of great value in differentiating strains of pneumococci or of streptococci. When the strain under examination has acquired, by successive passages through mice, the high degree of virulence required for the test, it is mixed with a specific serum and injected intraperitoneally into a mouse. If the strain is identical with that to which the serum is immune, the animal is "protected." As a standard, it is considered that 0.2 cc. of serum should, to establish identity of strains, protect against 0.1 cc. of virulent culture.

Serum treatment.—A therapeutic serum has been prepared against type I organisms but not for other groups. The serum has no beneficial effect on cases due to other types, and its value even in Type I cases is a matter of dispute. The serum is prepared by injecting horses first with dead organisms and subsequently with living ones. It is necessary to use a highly virulent strain and the organisms are injected intravenously. A satisfactory serum in amounts of 0.2 cc. should protect a mouse against 0.1 cc. of a culture the fatal dose of which is 0.000001 cc.

The serum is given intravenously in repeated doses of 50 to 100 cc. The average amount of serum required for a case is about 250 cc. Before giving the serum carry out intradermal and desensitizing tests. See Hypersensitiveness, p. 294.

Cecil and Larsen have recently treated 917 cases of lobar pneumonia with a pneumococcus-antibody solution prepared as directed by Huntoon. Horses are immunized against pneumococci, types I, II and III. The immune serum is treated with an equal volume of a heavy suspension of the specific organisms for one hour at 37°C., and centrifuged. The sediment is well washed with salt solution to get rid of all serum. The antibodies are then dissociated from the pneumococci (antigen) by emulsifying the sediment in salt solution containing 0.25% sodium bicarbonate and heating to 55°C. for about an hour. The mixture is then centrifuged, and the supernatant fluid, containing the antibodies in solution, is filtered through a filter candle.

The solution was given intravenously in doses of 50 to 100 cc. two or three times daily. In 20 to 30 minutes after administration, there occurred a severe reaction with chill and often severe cyanosis and dyspnoea. Of the cases treated, 38% were due to type I, 18% to type II, 16% to type III and 27% to type IV. For type I cases, the death rate was 13% as against 22% for controls. With type II and type IV cases, the death rate was somewhat reduced, showing the effect of cross protection with type IV infections. The solution had no effect on type III cases.

Prophylactic vaccines were tried extensively during the war, but the results could not be considered as proving the value of this form of protection against pneumonia.

Gram-negative Cocci—Neisseria Group

In this group are a number of organisms which show a diplococcal morphology, as a rule flattened out on the side toward the adjacent coccus—hence the descriptive terms coffee bean shape or biscuit shape. Two of these diplococci are of prime importance in disease, the *Gonococcus* and the *Meningococcus*. A third member of this group, *Micrococcus catarrhalis*, is chiefly of importance in that it may be confused with these two diplococci. Although it is considered as possibly producing a mild conjunctivitis or naso-pharyngeal catarrh (in the former infection it might be mistaken for the gonococcus—in the latter for the meningococcus) yet on the whole it may be considered as nonpathogenic.

The gonococcus might be confused with the meningococcus in connection with blood cultures but its isolation from the blood is a matter of extreme rarity and would probably be connected with a gonorrhoeal endocarditis or arthritis. On the other hand the infection with the meningococcus is now considered as primarily a bacteriemia.

Of the organisms, found in cultures made from the posterior pharynx, which are to be differentiated from the meningococcal types, may be mentioned *M. catarrhalis*, *M. flavus* (3 types), and *M. pharyngis siccus*. These confusing organisms are typically Gram-negative when first isolated but in subculture may show indications of being Gram-positive.

Gordon has considered the ability of the meningococcus-like organisms to grow at lower temperatures than the "normal" and "para" strains of meningococcus as being of differentiating value. The following table gives the fermentation reactions of the various Gram-negative cocci:

	Glucose	Maltose	Saccharose	Levulose
Meningococcus	+	+	−	−
Parameningococcus	+	+	−	−
Gonococcus	+	−	−	−
M. flavus I	+	+	+	+
M. flavus II	+	+	−	+
M. flavus III	+	+	−	−
M. catarrhalis	−	−	−	−
M. pharyngis siccus	+	+	+	+

Micrococcus flavus (*Neisseria flava*).—This organism is somewhat smaller than the meningococcus. The colonies have a yellowish center and adhere somewhat to the medium. There are three strains of this organism differentiated by fermentation reactions. It is well to remember that *M. flavus* will agglutinate with 1–50 normal horse serum so that in meningococcus agglutinations one should run a normal horse serum control, the meningococcus agglutinating only with immune serum.

Micrococcus pharyngis siccus (*Neisseria sicca*).—This is a small coccus which gives white colonies, which are firm, stick to the medium and do not easily emulsify. Growth takes place at room temperature.

Gonococcus (*Neisseria gonorrhoeae*). Neisser, 1879.—This organism is characteristically a diplococcus, the separate cocci being plano-convex with their flattened surfaces apposed (coffee bean shape). They are

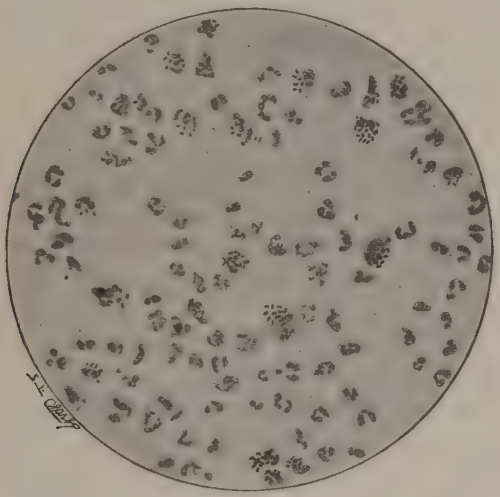


FIG. 21.—*Gonococcus*. Film from urethral pus. (*Coplin.*)

generally found grouped in masses of several pairs, most strikingly in pus cells or epithelial cells, but also found extracellularly. Except in the height of the disease, there is a great tendency for the organisms to show involution forms, so that instead of coffee bean-shaped diplococci we have round, irregular cocci.

It is therefore advisable in searching smears from cases of chronic gonorrhoea to continue the search of Gram-stained specimens until some fairly typical diplococci are found. There is nothing requiring greater discrimination than a diagnosis

from such a smear. It must be remembered that a latent or chronic gonorrhoea may persist for years practically unnoticed by the individual, yet the infection may be transferred even when a careful examination fails to show gonococci. Gonorrhoea in the female is responsible for much of the sterility and surgical conditions of the tubes and ovaries. During the war almost 6% of the recruits had venereal disease when taken in the Army and most of the cases were of gonorrhoea. At the commencement of a gonorrhoea the epithelial cells are abundant and gonococci are found adhering to them or lying free. Later on, at the acme of the discharge (the creamy, abundant discharge), it is in the pus cells we find them and they may be so abundant that 10 to 20% of the pus cells may contain them. In the subacute stage the epithelial cells, which practically disappear when the discharge is abundant, begin to reappear, and in the chronic stage the epithelial cells are the chief ones, and are the ones on which we find an occasional gonococcus, often distorted in shape. In gonorrhoeal ophthalmia the gonococci may show appearances in the conjunctival epithelium resembling the inclusion bodies of trachoma.

Diagnosis of chronic gonorrhoea.—The best method of diagnosis in cases of chronic gonorrhoea is to have the patient eat the stimulating food previously interdicted, to take active exercise and to have a sound passed. To obtain material for examination the glans penis should be washed and the patient who has presented himself with a full bladder should pass a portion of the contained urine. Next the prostate and seminal vesicles should be massaged with the patient standing but bent over and the penis pendant. The drops of discharge expressed by the massage should be received in a small Petri dish and finally the remaining urine should be passed into a sterile bottle. Smears and cultures should be made from the sediment of the two urinary specimens and from the secretions of the massaged prostate and vesicles.

The smears made from the resulting discharge or centrifuged urine will probably contain also any gonococci present in the urethra. In the female the favorite sites are the urethra and the cervix uteri. In municipal examinations it is customary to make two smears, one from the urethral meatus and a second from the cervix. The vagina is not a suitable soil for the development of gonococci. In female children they are most often found in the discharge of the vulvovaginitis, and such an infection in a children's hospital may spread among the little girls with frightful rapidity. It has, however, been questioned if the organism causing these epidemics is a true gonococcus. One of the greatest advances in preventive medicine was the Credé method of instilling a solution of silver nitrate into the conjunctival spaces of the newborn, thus preventing ophthalmia and blindness in those born of gonococcus-infected mothers. *Gram-stained smears from pus sediments of urine, especially in pyelitis or cystitis, may show coccoid forms of B. coli, which may be phagocytized and thus be reported as gonococci.*

In addition to the genital organs, the *Gonococcus* may at times invade and be isolated from the eye (gonorrhoeal ophthalmia) and the joints. Rarely it may cause endocarditis and septicaemia.

Cultural characteristics.—Grown upon hydrocele or ascites agar, or blood-streaked agar, or upon blood agar from man or the rabbit, the colonies appear as irregular, minute, dew-drop spots. By the second or third day the involution forms are abundant, and within four to ten days the culture will probably be found to be dead. Unless frequent transfers are made, it will be best kept alive on blood agar. The

organism grows best at 37°C., and will not grow below 25°C. (see p. 44). It will not grow on plain or glycerin agar or ordinary blood serum unless the transfer of considerable pus in inoculating the slants gives it a suitable culture medium. In material from joints, it is in the fibrin flakes that the gonococci are most apt to be found, if found at all. The gonococcus produces acid in glucose but not in maltose. Meningococci produce acid in both glucose and maltose.

Animals do not contract gonorrhoea. Even in monkeys urethral inoculations of gonococci are ineffective. The organism is killed in five hours by a temperature of 45°C. and speedily by drying. In moist smears of pus it may live for one or two days.

Complement fixation.—This test has considerable value in differentiating gonorrhoeal arthritis from that due to other causes. In preparing the antigen a number of different cultures should be used, since agglutination tests seem to show the existence of a number of strains of gonococci. Vaccine may be of some value in gonorrhoeal arthritis but in the ordinary case of specific urethritis there is no proof of its value. It is possible that the therapeutic results sometimes obtained with gonococcus vaccine are due, not to the specific nature of the vaccine, but to the reaction following the parenteral absorption of a nonspecific protein.

Diplococcus intracellularis meningitidis (*Neisseria intracellularis*). Weichselbaum, 1887.—This, the organism of epidemic cerebrospinal meningitis, is frequently termed the *Meningococcus*. It is a Gram-negative diplococcus, biscuit-shaped and, like the *Gonococcus*, chiefly contained in pus cells. It is also found free in the cerebrospinal fluid withdrawn from cerebrospinal fever cases. There is a greater tendency to variation in site and shape than is the case with the *Gonococcus*, which latter, in fresh material, shows a striking uniformity morphologically. The *Meningococcus* is at times not abundant. Early in the case, however, the picture may be similar to that of gonorrhoeal smears. The presence of Gram-positive cocci in the spinal fluid is almost always connected with a streptococcal or pneumococcal meningitis in which a fatal issue may be expected.

At times we may find the meningococci microscopically but not upon culturing the spinal fluid, or, we may obtain positive cultures where organisms were not found in the smear.

Rarely, it is impossible to demonstrate the presence of meningococci, especially where the case may show a fairly clear fluid (with only slight flocculent material) instead of the turbid one usually withdrawn.

Cultural characteristics.—On blood serum the colonies appear after twenty-four to forty-eight hours as discrete, very slightly hazy colonies, about 2.5 mm. in diameter. On serum agar, as ascites or hydrocele agar, they grow best and show as faint bluish colonies about 1 to 2 mm. in diameter. They are larger than *Streptococcus* or

Pneumococcus colonies. Unless considerable cerebrospinal fluid is transferred with the inoculating loop, they do not grow on plain agar. They will grow at times on glycerin agar. The best medium for plate cultures from carriers is blood agar. The haemolytic and green-appearing colonies of streptococci and the greenish pneumococcus colony are readily differentiated. The meningococcus colony is slightly opaque in the center and rather clear at the periphery. While laked blood agar plates have the advantage of a lens effect with transmitted light yet on the whole the more opaque blood agar is to be preferred. The organism is very sensitive to light, cold and drying. It ferments glucose and maltose but not lactose or saccharose, and grows only at blood temperature, thus distinguishing it from the *M. catarrhalis* which will not ferment any of these sugars, and grows at room temperature. The meningococcus is scarcely pathogenic for laboratory animals, with the exception of the mouse and guinea pig, with which intraperitoneal and intradural injections, but not subcutaneous ones, give results. The cultures die out very rapidly, so that it is necessary to make transfers every one or two days. Stock cultures are best kept on ordinary bouillon containing 0.5% agar, or on blood agar or egg-yolk slants.

Presence in naso-pharynx.—The *Meningococcus* has been isolated from the nasal secretions of patients, but on the whole nasal cultures are negative, the organisms preferring the posterior naso-pharynx. The possibility of the organisms found being the *M. catarrhalis* must always be considered. Of epidemiological importance is the detection of the healthy carriers by the examination of the nasopharyngeal secretions of those known to have been in contact with a patient. To examine such persons, introduce a bent wire applicator with sterile cotton tip past the soft palate so as to get material from the naso-pharynx. The material should be immediately inoculated on blood or serum agar and quickly put in the 37°C. incubator. The plates should be warm when inoculated and kept at 37°C. until placed in the incubator.

Flexner has shown that in monkeys, which are susceptible to the disease, injections of cultures of meningococci into the spinal canal is followed by migration of the cocci to the nasal cavity, where they are found both free and in phagocytes.

Epidemiology.—The old view concerning transmission was that the organism, by whatever way transferred, gained lodgment in the naso-pharynx and thence migrated by the lymphatics to the base of the skull and meninges. The meningococcus, however, has very slight resistance to sun or drying, so that its aërial transference is at least doubtful. At present our views are that infection takes place through direct contagion, and that cerebrospinal fever is primarily a blood infection, the meningococci localizing later in the central nervous system. In fact, the infection not rarely remains confined to the blood stream, apparently not invading the central nervous system at any time in the course of the illness. Several cases have been reported where, with a high leukocytosis, the cocci have been found in about 35% of blood cultures where 5 to 10 cc. of blood were employed, and even in the

polymorphonuclears in blood smears. It is because of the almost invariable infection of the blood stream that we now habitually make blood cultures as well as spinal fluid ones, and inject serum intravenously as well as intrathecally.

Experience during the late war indicates that most adults are immune, but there is no method of determining susceptibility comparable to the Schick test with diphtheria.

Serum treatment.—By the use of initial injections into horses of killed polyvalent cultures, followed by alternate injections of living diplococci, then seven days later of an autolysate made from different strains; seven days later again injecting living diplococci—thus alternating material every week, an antiserum of value has been

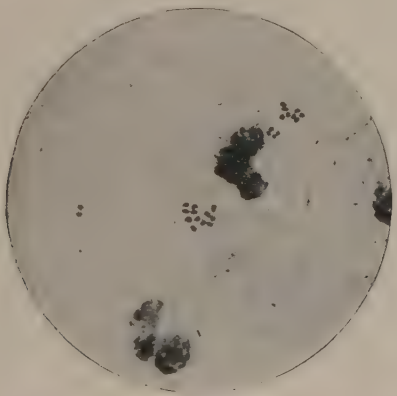


FIG. 22.—*Diplococcus intracellularis meningitidis* and pus cells. (× 1000) (*Williams.*)

obtained by Flexner. The immunization formerly required about one year, but recently it has been possible greatly to shorten this period. In producing this polyvalent therapeutic serum, the makers should obtain cultures of every strain found to be associated with cases of the disease. The serum should agglutinate normal and para types in 1 to 1200. Monovalent sera, produced by the injection of animals with a single strain, are in use, but only for ascertaining the strain involved; they are not employed therapeutically, since serum must be administered before strain determination can be completed.

To administer serum, withdraw spinal fluid until pressure is relieved and fluid comes out by drops at the rate of 4 or 5 per minute. Leaving needle in place, inject serum in amount equal to about one-half the spinal fluid removed. Repeat injection according to symptoms. In intravenous administration, use 50 to 100 cc.

Types of meningococci.—Dopter first called attention to the existence of two types which differed immunologically—the normal and the para strains. The Rockefeller

Institute recognizes these two types and also irregular types. The English recognize 4 types—I, II, III and IV. Type I corresponds to Flexner's para strain and type II to his normal one. Types III and IV are irregular ones.

Holm and Davison state that Gordon's types I and III correspond to the Rockefeller "para" strains and to Pasteur type A. The Gordon II and IV correspond to the Rockefeller "normal" strains and to Pasteur type B. Pasteur types C and D are rare.

Gordon notes that there is somewhat close affinity between his types I and III and between types II and IV. He also notes that chronic carriers tend to continue to carry the same type organism. As to frequency of types in cases in England he has found the following: For type I, 40%; type II, 45%; type III, 10 and type IV, 5%. The types found in cases and in carriers show a close correspondence.

For diagnosis, make blood cultures, and smears and cultures from cerebrospinal fluid.

Smears should be made from sediment obtained by centrifugalization of the spinal fluid. In cerebrospinal meningitis, the polymorphonuclears predominate; in tuberculous meningitis, the lymphocytes, and in that due to streptococci or pneumococci Gram-positive organisms are found. For final diagnosis of meningococci, as well as for type, agglutination reactions are necessary. See Agglutinins, p. 245.

As a rule the organisms are phagocytized by the polymorphonuclears, but they may be extracellular. Occasionally they cannot be demonstrated owing to their being subject to autolysis; hence when a fluid presents a predominance of pus cells but contains no organisms, the infection is almost surely meningococcic—cases due to streptococci or pneumococcus practically invariably showing organisms. At times there does not appear to be any relation between the number of phagocytic polymorphonuclears and the severity of the case.

Carriers.—In the examination of incoming recruits for the military services it is not unusual to find as many as 2% showing meningococci in nasopharyngeal cultures. Persons in contact with cases of meningitis or with well carriers may show a high incidence—10%, or even higher.

Most cases cease to be carriers within a few weeks and well carriers tend to follow the same rule. It is a question whether treatment of carriers is of much avail. Warm weather, especially when combined with tent life, tends to cause the organisms to disappear from the naso-pharynx. The carrier percentage in military barracks may often be kept down by suitable spacing between cots. There are some cases which persist in the carrier state in spite of any treatment or environment. These "persistent" carriers may give an occasional negative culture, but if we demand 3 or 4 successive negative cultures, at intervals of five days, before pronouncing the man free from his infection, the carrier period may be found to persist for months.

It is striking the rarity with which such carriers come down with the disease, but resistance-reducing factors, as colds, excessive fatigue, chilling, etc. may cause them to develop the disease.

The English naval statistics indicate that the disease almost always develops in those who have previously shown negative cultures. American statistics fail to show the same phenomenon. Young children are most susceptible to meningitis and contact with a carrier is more dangerous for them than for an adult. Carriers are vastly more common in winter than summer and the disease is peculiarly a disease of cold, inclement weather.

Micrococcus catarrhalis (*Neisseria catarrhalis*) Seifert, 1890.— This organism has been specially studied by Lord. It resembles the *Meningococcus* strikingly and can be differentiated only by agglutination and cultural procedures. It grows on plain agar and at room temperature. It not only occurs in the nasal secretions of healthy people, but appears to be responsible for certain coryzas and bronchial affections resembling influenza. It is responsible also for certain epidemics of conjunctivitis.

Original cultures may show only slight growth whereas subcultures prove luxuriant. The colonies are larger, more opaque, and have a more irregular wavy border than the round colonies of the *Meningococcus*. The colony is usually easily picked up from the plate with the loop but does not emulsify readily. *M. catarrhalis* grows best at 37°C. but also fairly well at 22°C. after several days, while the *Meningococcus* requires body temperature. It does not ferment with acid production any of the sugars.

CHAPTER VI

STUDY AND IDENTIFICATION OF BACTERIA—SPORE-BEARING BACILLI. KEY AND NOTES

A. **Grow Aerobically.**—Mostly saprophytes. Generally liquify gelatin and digest coagulated blood serum. Often occur in long chains and form rhizoid colonies. Usually Gram-positive. Form of rod usually not greatly changed at sporulation. (Type species *Bacillus subtilis* Cohn.)

(a) Pathogenic forms.

(1) Bacillus anthracis. Nonmotile rods with square cut to concave ends, occurring in long chains. Central spores.

(b) Nonpathogenic forms. Usually motile, having central or excentric spore. Facultative aerobes.

(1) Bacillus subtilis group. Organisms of this group are commonly found in intestinal contents, soil, water and milk. Seventy-five different species described, showing slight cultural differences. Many produce pigment.

NONPATHOGENIC SPORE-BEARING AEROBES ON AGAR

Modification of table of Gruner and Fraser

Surface dry	Gray-white	*B. subtilis* (motile).
		B. ellenbachiensis (nonmotile).
	Edges feathery	*B. mycoides*.
Surface gummy	White	*B. mesentericus*.
Surface moist and exuberant	Yellow	*B. ruminatus*.
	Dirty gray	*B. graveolens*.
	White and wrinkled.	*B. vulgatus*.

NOTES.—*B. subtilis* has square ends and central spores, not causing bulging.

B. vulgatus is long and slender. Slightly oval spores.

B. mesentericus varies. Usually short with rounded ends and central bulging spores.

B. ellenbachiensis has rounded ends, oval spores and shows granule formation (beaded)—resembling diphtheroids.

All of these grow well at room temperature, optimum 30°C.

B. **Grow Only Anaerobically.**—Producing clostridium (spores) forms. Often parasitic and many elaborate exotoxins.

(1) Motile. Rods swollen at sporulation.
  (a) Spores oval, central or excentric.
    (1) Clostridium chauvei. (Called the bacillus of symptomatic anthrax; cause of black leg or "quarter evil" in cattle.)
    (2) Clostridium oedematis-maligni (*Vibrion septique* the cause of malignant oedema).
    (3) Clostridium oedematiens. (Gas-oedema bacillus of Aschoff.)
    (4) Clostridium botulinum, Type A. (The cause of botulism. Filtrate toxic for guinea pigs and chickens.)
    (5) Clostridium botulinum, Type B. (Differs from Type A in toxicity. Non-toxic for chickens. Exotoxin not neutralized by Type A antitoxin.)
    (6) Clostridium histolyticum. (Isolated from war wounds. Produces necrosis in tissues.)
    (7) Clostridium sporogenes. (Isolated from intestinal contents. Indicates faecal pollution of water.)
    (8) Clostridium fallax. (*B. fallax*. Feebly pathogenic.)
  (b) Spores terminal or subterminal. Spherical or nearly so.
    (9) Clostridium tetani. (Cause of tetanus. Forms highly poisonous exotoxin.)
  (c) Spores oval or elongated.
    (10) Clostridium tertium. (Isolated from war wounds.)
(2) Nonmotile. Rods not swollen at sporulation.
  (a) Spores central or excentric. Encapsulated.
    (1) Clostridium welchii. Types I, II, III, IV. (Cause of gas gangrene. Types differentiated by fermentation reactions.)
    (2) Clostridium egens. (Similar to *welchii*.)

Spore-bearing Aerobes

Bacillus anthracis (Pollender discovered 1849. Davaine recognized nature 1863. Koch proved 1876).—Of the aerobic spore-bearing bacilli this is the only one of particular medical importance.

Anthrax is an important disease in domesticated animals, especially sheep and cattle. The characteristic postmortem change in animals is the greatly enlarged, friable, mushy spleen. Man is much less suceptible than these animals, but is more so than the goat, horse, or pig. The Algerian sheep has a high degree of immunity, as has the white rat. The brown rat is quite susceptible as are also guinea pigs, mice and rabbits. The disease in man chiefly occurs among those working with hides, wool, or meat of infected cattle. Many infections have occurred from the use of shaving brushes. The new brushes are especially dangerous and the organism has been cultured from such brushes. Thorough washing in hot soapy water seems to free the brush of its infection by mechanical means. Horsehair from China and Thibet seems the chief source of infection. The two chief types in man are: 1. Malignant pustule and 2. Woolsorter's disease. An intestinal type is also recognized.

Malignant pustule.—Results from the inoculation of an abrasion or cut; thus it frequently shows on the arms and the backs of those unloading hides. It first appears as a pimple, the center of which becomes vesicular, then necrotic.

A ring of vesicles surrounds this central eschar, and a zone of congestion lies outside the vesicles. The lymphatics soon become inflamed as well as neighboring glands. If death occurs in a case in which the pustule has not been excised, there is not found postmortem the enlargement of the spleen and the abundance of bacteria in the kidneys seen in animals. Man seems to die from toxaemia rather than a septicaemia. A few cases have been reported of positive blood cultures. These cases usually die but such a case has recently been reported by Graham in which intravenous injections of anti-anthrax serum were successful.

FIG. 23.—Anthrax bacilli. Cover glass has been pressed on a colony and then fixed and stained. (*Kolle and Wassermann.*)

FIG. 24.—Anthrax bacilli growing in a chain and exhibiting spores. (*Kolle and Wassermann.*)

Woolsorter's disease.—In woolsorter's disease there are great swelling and oedema of the bronchial and mediastinal glands. The lungs show oedema, which about the bronchi is haemorrhagic. It is considered that the disease arises from the inhalation of anthrax spores.

Cultural characteristics.—The bacillus is 5 to 8μ by 1 to 1.5μ and nonmotile. In cultures it has square-cut or concave ends and is often found in long chains, but in the blood of an infected animal the bacilli are in quite short chains and the ends may be slightly swollen. A capsule may be noted in preparations from the animal body. It is Gram-positive. *B. anthracis* grows well on all media and it rapidly

liquefies gelatin; about the time liquefaction begins in a gelatin-plate colony, we have the medusa-head appearance which is quite characteristic for anthrax. There is a slow acid and coagulating effect on milk. Potato is the best medium for spore development. Spores develop best at a temperature of 30°C. and do not form at temperatures above 43°C. They are oval and centrally placed. They stain with difficulty.

There is also an aerobic spore-bearing bacillus called *B. anthracoides*, which differs morphologically from anthrax solely in its rounded ends in culture. Its growth is more rapid and it liquefies gelatin more energetically.

Atrium of infection.—Stiles thinks that animals are infected by eating the bones of animals which have died of anthrax, cutting buccal mucous membrane, and so becoming infected. Spores do not form in an intact animal body, but they do form

FIG. 25.—*Bacillus anthracis* in blood of rabbit. (*Coplin.*)

after a postmortem or the disintegration of the body by maggots. For this reason it is better at autopsy not to open up the body of the animal, but to make the diagnosis by cutting off an ear. Another view concerning infection is that the spores are swallowed by the animal, vegetative forms develop in the alimentary tract and invade the body through the intestinal wall. Dried spores will live for years and will withstand boiling temperature for hours.

Immunity.—The explanation of the immunization of animals is obscure, as injection of dead organisms seems to have no effect. There is also much question as to the value of the antiserum. In vaccinating animals against anthrax, Pasteur used two vaccines, the first attenuated fifteen days at 42.5°C., the second, attenuated for only ten days, given twelve days later. At these higher temperatures only vegetative forms develop. Prolonged incubation lowers virulence so that this is lost for rabbits, but not for guinea pigs or white mice. A longer treatment destroys the virulence for guinea pigs also and eventually that for mice.

Various bacteria, especially *B. pyocyaneus*, show marked antagonism to *B. anthracis*. Pyocyanase digests the anthrax bacillus and has been used to cure animals infected with anthrax.

Diagnosis.—In taking material from a malignant pustule before excision, be careful to avoid rough manipulations, lest bacteria be expressed into the circulation. Make cover-glass preparations, staining by Gram. Make culture on agar. Blood cultures are usually positive, if at all, only late in the disease. Inoculate a guinea pig or a mouse subcutaneously.

The guinea pig dies in about forty-eight hours and shows an oedematous gelatinous exudate at site of inoculation. The blood is black and swarms with anthrax bacilli. It is the best example of a bacteriaemia. The mouse should be inoculated at the root of the tail.

Serum treatment.—In cases where the infection becomes septicaemic instead of localized, the outcome is usually fatal. In such cases one should inject anti-anthrax serum intravenously, in doses of 50 to 75 cc. In malignant pustule it is advisable to inject the serum in the subcutaneous tissues surrounding the lesion. The anthrax antiserum is made by simultaneous inoculation of animals with cultures of *B. anthracis* and antiserum. The best animal to use is the sheep and a preliminary vaccination by Pasteur's method is to be carried out. It is usually given in doses of 25 to 50 cc.

Spore-bearing Anaerobes

There are four very important pathogens in this group—that of gas gangrene (*B. welchii*); that of malignant oedema (*Vibrion septique*); that of botulism (*B. botulinus*), and the organism of tetanus (*B. tetani*).

The *B. sporogenes* is of importance in connection with indications of faecal contamination of water. In connection with *B. welchii*, there is some question as to whether the extensive oedema produced by it may not usually be from a terminal or cadaveric infection. At any rate necrotic material seems necessary for its development.

It should be stated that our knowledge of the differential cultural characteristics of anaerobes is unsatisfactory. The exact methods which are in use for aerobes have not been applied to anaerobic organisms.

Cultivation of Anaerobes. *Novy Jar.*—Probably the apparatus giving the most perfect anaerobic conditions is the Novy jar, in which the air has been replaced by hydrogen. The difficulties attending the method are:

1. Unless a special apparatus (Kipp's) is at hand, there may be difficulty in preventing the sulphuric acid from frothing over when poured on the zinc. It should, at first, be added in small quantities at a time—well diluted (1 to 6).

2. Various wash-bottles are required: One containing silver nitrate solution for traces of AsH_3 and one with lead acetate for H_2S and another with pyrogallic acid and caustic soda for any oxygen that may come over.

3. Mixtures of hydrogen and air explode. Consequently, in determining whether all air has been expelled and in its place an atmosphere of hydrogen exists, it is necessary to see if the escaping gas burns with a blue flame. Unless this is collected in a test tube and examined, an explosion may result.

4. Except in a large laboratory, where the apparatus is set up and ready for use, too much time would be required.

5. Simpler methods appear to give as good results.

NOTE.—Hydrogen gas is now easily obtained in cylinders and with a reducing valve is available for filling culture jars.

Tarozzi's method.—In this method, pieces of fresh sterile organs are added to bouillon. Pieces of kidney, liver, or spleen are best suited. After adding the tissue the media may be heated to 80°C. for a few minutes without interfering with the anaerobic-producing properties of the fresh tissues. This method is practically the same as that recommended by Smith (see Tetanus). This is also a feature of Noguchi's method of culturing *Treponema pallidum.*

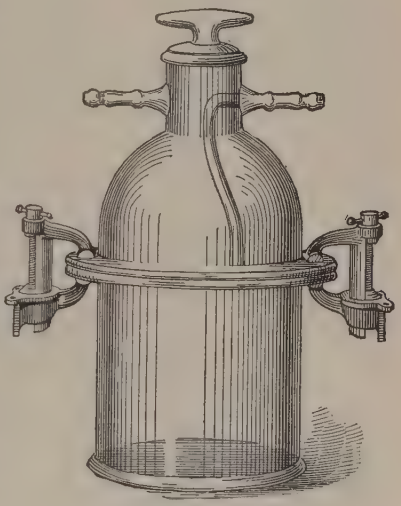


FIG. 26.—Novy jar.

For method for partial oxygen tension see pages 44, 82.

The method of Liborius.—In this it is necessary to have a test tube containing about 4 inches of a 1% glucose agar. Glucose acts as a reducing agent and furnishes energy. It is convenient to add about 0.1% of sulphin digotate of soda, the loss of the blue color at the site of the colony enabling us to distinguish it. The tube of agar should be boiled just before using to expel remaining oxygen from the tube. Then rapidly bring down the temperature to about 42°C., by placing the tube in cold water, and inoculate with the material to be examined. A second or third tube may be inoculated from the first, just as in ordinary diluting methods for plate cultures. Having inoculated the tubes, solidify them as quickly as possible using tap water or ice-water. The anaerobic growth develops in the depths of the medium. Some pour a little sterile vaseline or paraffin or additional agar on the top of the

medium in the tube as a seal from the air. Others have recommended the inoculation of some aerobe, as *B. prodigiosus*, on the surface. This latter method is not advisable. A deep stab culture is often sufficient.

The same technique can be applied to gelatin cultures for anaerobes, pouring in at the completion of the inoculation an inch or so of melted glucose agar to act as a stopper for the gelatin below.

The method of Buchner.—In this method 1 gram each of pyrogallic acid and caustic potash or soda for every 100 cc. of space in the vessel containing the culture is used to absorb the oxygen. It is convenient to drop in the pyrogallic acid; then put in place the inoculated tubes or plates; then quickly pouring in the amount of caustic soda, in a 10% aqueous solution, to immediately close the containing vessel. A large test tube in which a smaller one containing the inoculated medium is placed, and which may be closed by a rubber stopper, is very convenient. A good rubber-band fruit jar is satisfactory. A desiccator may be used for plates. An excellent method for obtaining anaerobic conditions with plates, either in a desiccator with the pyrogallic acid and caustic soda, or—less satisfactorily—in the open air, is to sterilize the parts of the Petri dish inverted; that is, the smaller part is put bottom downward in the inverted cover (as one would set one tumbler in another). Then, in using, unwrap the Petri dish, lift up the inner part, pour in the inoculated medium into the upturned cover. Then immediately press down the inner dish, spreading out a thin film of the medium between the two surfaces.

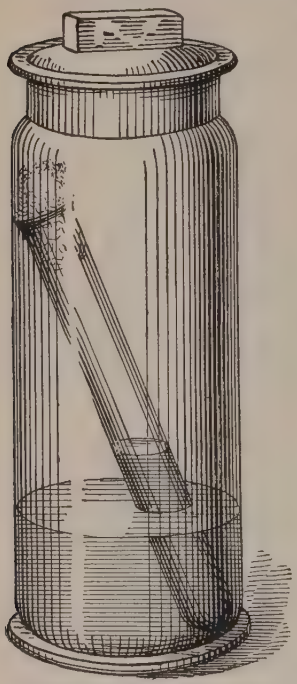


FIG. 27.—Arrangement of tubes for cultivation of anaerobes by Buchner's method. (*Mac Neal.*)

Zinsser's method.—Zinsser has originated a very satisfactory method for plate cultures of anaerobes, which is shown in Fig. 8.

Secure two small crystallizing dishes, about 3 and 4 inches in diameter by 1 inch in depth and sterilize. Pour the inoculated agar into the smaller of the dishes, or smear the surface of poured glucose agar with the material to be plated out. In the bottom of the larger dish place the dry pyrogallic acid, then invert the smaller dish with the agar surface over it. Quickly pour a 5% solution of caustic soda into the space separating the sides of the inverted smaller dish and the upright larger dish, to a depth of ½ inch, and while it is dissolving the pyrogallic acid, very speedily superimpose paraffin oil on the soda solution to make an air-tight seal.

J. H. Wright's method.—Make a deep stab culture in glucose agar or gelatin, preferably boiling the media before inoculating. Then flame the cotton plug and press it down into the tube so that the top lies about three-fourths of an inch below the mouth of the test tube. Next fill in about one-fourth of an inch with pyrogallic

acid; then add 2 or 3 cc. of a 10% solution of caustic soda, and quickly insert a rubber stopper. This method is one of the most convenient and practical, and is to be strongly recommended.

Vignal's method.—In this, a section of glass tubing (¼ in.) is drawn out at either end, so that there remain two narrowings of the lumen with an intervening section of full diameter. One end, which is to be used as a mouth-piece, is plugged with cotton; the other is broken off at the point of constriction. The liquid agar or gelatin is then inoculated and the medium drawn up into the tube by suction with mouth or better with a rubber bulb. In a very small flame the capillary narrowings are sealed off, and we have inside the tube very satisfactory anaerobic conditions. To get at the colonies, file and break the tube at the desired point.

NOTE.—To obtain material for examination and isolation in pure culture from the deep agar stab-tube, it is best to loosen the medium at the sides of the tube with a heated platinum spud or a flattened copper wire. Then shake the mass out into a sterile Petri dish. It is dangerous to break the tubes with a hammer as some do. With those anaerobes which produce gas in glucose agar the split in the column of medium enables one to introduce a fine sterile capillary pipette to the site of a colony and by releasing pressure on the rubber bulb to draw up into the tip of the tube material for investigation.

Method of McIntosh and Fildes.—This, or one of its modifications, is now regarded as giving more satisfactory anaerobic conditions than do other methods. The method depends on the action of hydrogen gas on platinized asbestos. Palladium chloride is probably better. Hydrogen gas is allowed to enter the jar, passing over the platinized asbestos wool. The oxidation of the hydrogen removes the oxygen. The process is not devoid of danger unless great care is taken, since an explosion may occur if the hydrogen is run in too rapidly.

A combination method.—As shown in the illustration in Fig. 12, I have been combining various methods so that very satisfactory anaerobic conditions are obtained. First, a deep agar stab of freshly sterilized glucose agar is made and inoculated. This tube and the proper amount of pyrogallic acid are then deposited in a salt mouth bottle. The rubber stopper with the glass and rubber tubing is then firmly pushed in and connection made with a filter pump.

In five to ten minutes almost all the air will be exhausted when the Hoffmann clamp is screwed up tight and the bottle disconnected from the vacuum pump. The glass tubing end is then inserted into a graduate holding 10% caustic soda solution, the Hoffmann clamp unscrewed, and the necessary amount of caustic soda having been run in, as noted under Buchner method, we again close the screw clamp and incubate. This method may be used with a flat-sided culture flask in which the medium, such as glucose agar or blood agar, has been allowed to solidify on one side. This culture-medium surface is then inoculated and the flask placed with the culture-medium surface uppermost. After exhausting the air, run in first a 50% solution of pyrogallic acid and next 10% caustic soda solution.

General Considerations of Pathogenic Anaerobes

It is now generally accepted that the literature describing anaerobes prior to about 1916 is untrustworthy as many of those investigating these organisms were dealing with mixed cultures.

As pointed out by Kendall, *B. sporogenes* is very apt to be present in supposed pure cultures of other anaerobes, and the presence of this contaminant will explain conflicting statements as to liquefaction of gelatin and other proteolytic activities on the part of organisms we now know to have no such power. Kendall regards the selection and inoculation of a single spore, picked up by the Barber technique, as essential in the satisfactory isolation of the pathogenic anaerobes. Attempted isolation of a single vegetative bacillus is attended with much less success.

With the exception of *B. botulinus*, the pathogenic anaerobes are common inhabitants of the intestinal tract of man and other animals—hence their frequency in fertilized soils. The spores of most of these anaerobes develop in the intestinal lumen and in this resistant stage may remain viable for years in the soil. In Flanders the soil was heavily contaminated and the clothing of the soldiers, spattered with the spore-containing mud, furnished infectious material ready to be inoculated by shells or other agents. Some of these anaerobes as the gas bacillus and *Vibrion septique*, lack the proteolytic action of such active digestants of blood serum and coagulated milk as *B. sporogenes* and *B. histolyticus*.

Dean and others working with material from gas gangrene wounds found an egg broth made by shaking up the white and yolk of one egg in 300 cc. water a most excellent culture medium. This medium was tubed and sterilized, after which it was liberally inoculated with material from the wounds. After inoculation the tube was heated to 80°C. for one-half hour and then incubated anaerobically. The gas bacillus was present in such tubes in abundance after two or three days of incubation while the tetanus bacillus appeared only after a prolonged period—seven to ten days.

In case of the tetanus bacillus they tried various methods of bacteriological diagnosis. The examination of smears from wounds was unsatisfactory in the search for "drum-stick" spores; in broth cultures the spores were not present until after several days, and in mixed cultures it was difficult to be sure that the terminal spores were those of tetanus and not those of other terminal-spore organisms. The best method, they found, was to inject guinea pigs in the subcutaneous tissues of the left chest with 1 or 2 cc. of mixed broth culture. In two or three days stiffness of the left forelimb was observed, it soon becoming quite stiff and extended. The spasm extended to other limbs and death occurred in one or two days. There was no evidence of marked inflammation at the site of inoculation.

There have been a number of cases of delayed tetanus often associated with operations done a month or two after the original wound infection so that it is recommended to give antitetanic serum before operation on such cases.

Bacillus chauvei (*Clostridium chauvei*).—This is an anaerobic spore bearer, called the bacillus of symptomatic anthrax, blackleg or quarter-evil, which causes a rapidly developing emphysematous swelling, with a dark color, of the thighs. It has bulging slightly oval spores at one

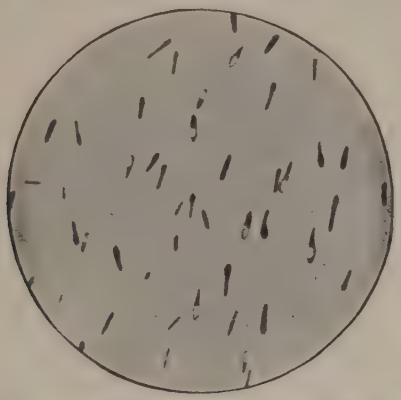


FIG. 28.—*B. chauvei.* Symptomatic anthrax (Rauschbrand) bacilli showing spores. (*Kolle and Wassermann.*)

end, but they are not distinctly terminal as with tetanus spores. It affects sheep and cattle but not man. It is a soil organism like those of tetanus and gas gangrene.

It is difficult to separate this organism from *Vibrion septique* but as the latter alone is concerned in human infections this difficulty is important only for veterinarians. Robertson gives as differentiating points the fermentation of salicin but not saccharose by *Vibrion septique* while *B. chauvei* ferments saccharose but not salicin. *B. chauvei* does not exhibit the gliding-serpent chains which are such a feature of fresh liver emulsion and blood preparations from guinea pigs infected with *Vibrion septique.* Many have reported *B. chauvei* as Gram-negative.

Vibrion septique (*Clostridium oedematis-maligni*) Pasteur, 1877.—This organism was isolated by Pasteur from an animal supposed to have died of anthrax. Later, Koch isolated an anaerobe from garden soil which he named *B. oedematis maligni* although he regarded it as identical with Pasteur's *Vibrion.* He however reported it as liquefying gelatin, and, since the *Vibrion septique* has no such

action, it seems certain that he was not dealing with that organism. Koch's bacillus is now consired to be indentical with *B. sporogenes*.

Morphology and cultural characteristics.—*Vibrion septique* is a rather narrow bacillus with rounded ends measuring on the average 6 × 0.4μ. It is motile, and, in a fresh specimen of the blood of a guinea pig dying of the infection, appears in long undulating chains which move among the blood cells as serpents in the grass (Pasteur). In wet preparations from the liver of the guinea pig, these serpent-like forms are common. The bacillus forms oval spores which usually lie between the center and the end, but rather characteristic is the formation of a spindle-shaped organism with a large central spore and shrunken ends (lemon shape). Sporulation does not seem to take place in the body of the host. The organism is Gram-positive. It ferments milk with gas production, but less actively than does the Welch bacillus. It has little proteolytic action, not liquefying but only softening gelatin, and not acting on blood serum. It ferments glucose and lactose, but not saccharose. A similar anaerobe, *B. chauvei*, ferments saccharose. The colonies are filamentous and spreading. The odor of culture is sour or rancid—not foul.

Pathogenicity.—The organism was occasionally found in war wounds, usually in association with other anaerobes. It produces a soluble toxin which causes oedema and necrosis of tissues and contains an haemolysin. This toxin, like that of the gas bacillus, seems not to require a period of incubation, but excites the formation of an antitoxin. A rather large dose is required to kill an animal. The lesions of animals dying of the infection resemble those produced by the gas bacillus, but the *Vibrion* may be distinguished from the gas bacillus by its motility. In man the infection has been noted only in war wounds, but in herbivorous animals it may occur with or without wounds. *Vibrion septique* has been isolated from milk.

B. botulinus (*Clostridium botulinum*) Van Ermengem, 1896.—This is the organism which produces botulism, a form of food poisoning. It is a spore-bearing anaerobe and must not be confused with another organism, a non-sporing aerobic bacillus, associated with meat poisoning—the *B. enteritidis* of Gärtner. The botulinus spores, which are oval or subterminal, are very resistant, some surviving autoclave temperatures applied for several minutes.

Interest in botulism in the U. S. has been stimulated by fatalities from eating ripe olives. Instead of meat products being implicated, as is usual in Europe, most American infections have resulted from eating canned string beans, asparagus, corn, apricots, olives and cheese. In his work Weinzirl has noted that commercial canned products are more likely to be safe than food preserved in the household. Most human infections in the U. S. can be traced to California. Burke has isolated the organism in different localities of California from such material as leaves of bean plants, moldy hay, cherries and spiders. She is of the opinion that the organism may be disseminated by spiders or other arthropods. *B. botulinus* is found in virgin soil as well as that under cultivation.

In botulism the meat becomes infected after the animal has been slaughtered; in Gärtner meat poisoning the meat was already infected at the time of slaughter—it was from a sick animal. Thorough cooking of the meat protects against botulism but not certainly against Gärtner meat poisoning. The botulism toxin is destroyed in 30 minutes at 80°C. and in shorter time at boiling temperature. It is important to remember that the endotoxins from autolysates of *B. enteritidis*, another important food poisoning organism, are not destroyed by a brief exposure to 100°C.

In botulism, dysphagia, ocular paralyses and cardiac and respiratory symptoms (medulla) are observed. There is no fever, and consciousness is preserved. These clinical signs are due to the absorption

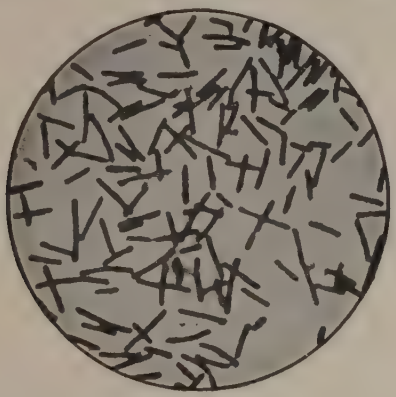


FIG. 29.—Bacillus of botulism. (*Kolle and Wassermann.*)

of a toxin, resembling those of diphtheria and tetanus in being soluble and extracellular, but unlike them in that it is elaborated solely outside the body, is ingested preformed in food substances and is not destroyed by putrefaction or by alimentary tract digestion. Botulism is not an infection, but a true intoxication.

An antitoxin which it is stated has therapeutic value in botulism has been prepared in the usual way by Kempner. He used goats for immunization. We now know that antiserum for type A organisms has no effect when the intoxication is from type B organisms. Consequently a polyvalent serum should be used. Since the serum does not seem to be of much value when symptoms have fully developed, it should be used early. Without serum treatment death occurs in about 40% of cases and takes place in between twenty-four and forty-eight hours.

The bacillus has been isolated from sausage and ham as well as from various canned vegetables. It is a rare cause of food poisoning, most of such cases being the result of paratyphoid or enteritidis infections. It is a large bacillus—5 to 7μ by 1μ—and when sporulating has the spore between the center and the end. It is slightly

motile and stains by Gram. It produces gas in glucose media. It does not coagulate milk, which is not a favorable medium. Van Ermengem stated that it liquefied gelatin, but Kendall contradicts this, asserting that liquefaction is not observed in pure cultures. It grows best at 22° and only slightly at 37° -hence it is dangerous only from its soluble toxin, the bacilli not developing to any extent in the body.

For this reason botulism patients are not a source of danger; it is infected food alone which causes the disease. On the contrary, where meat poisoning is due to the Gärtner or paratyphoid group, infection may take place from the patient's discharges.

Botulism in America.—As is now generally known, botulism poisoning may occur from eating infected vegetables as well as meat. In 18 reports of botulism poisoning in the U. S. 11 were traced to canned fruits or vegetables. Since the toxin is destroyed by moderate heat, cooking food immediately before eating removes the danger from botulism, but such articles are sometimes eaten raw as in salads.

Jennings and others have reported 8 cases of botulism poisoning with 6 deaths, from eating canned ripe olives. The onset of symptoms was within 24 hours in 2 cases, 60 hours in one case and 70 hours in four cases. There was no fever and the mentality was clear throughout the disease until about the end. Vomiting was pronounced in 3 cases and absent in 4 cases. The constant manifestations were visual disturbances, generally followed by difficulty in swallowing. The cranial nerves involved were the 3rd, 4th, 6th, 9th, and 10th. The 1st, 5th and 8th and, except in one case, the 7th, were not involved. Apparently antibotulinus serum must be given early and probably in doses of 50 to 100 cc. as they had no results from 25 cc. intravenously in one case. Statistics covering 180 cases reveal 122 deaths—a mortality of about 70%.

When the toxin is introduced, it requires a period of incubation of twelve to twenty hours. Symptoms of gastrointestinal disorder may come on shortly after the ingestion of the toxin-containing food, these however are not the specific manifestations, as are the eye symptoms, etc.

An important point is that ham or canned vegetables may not appear decomposed and yet contain many bacilli and much toxin. It is a very potent toxin—as little as $^1/_{1000}$ cc. or even 1/1,000,000 cc. with some strains may kill a guinea pig.

Types of B. botulinus.—Two strains of the botulism organism are now recognized—types A and B. The antitoxin of one type fails to neutralize the toxin of the other. Type A organisms seem more common in Pacific Coast outbreaks and type B in the East.

Examination of food.—To ascertain the presence of the toxin, inject an infusion of the ham or sausage, or liquid part of canned olives or vegetables, suspected of causing the poisoning, intraperitoneally into a guinea pig, and characteristic pupillary symptoms with death by cardiac and respiratory failure will result.

Cultures may be made in glucose agar. The culture is disrupted by gas. Incubation at room temperature and in the dark is necessary. There is a sour or rancid odor. The characteristic point is the production of a powerful soluble toxin which produces symptoms when no bacilli are present. Chickens are susceptible to poisoning with type A toxin but not that of type B. In the chicken it produces a paralysis with weakness of the neck (limber neck) and death in 24 hours. In a suspicious human case in a house always note condition of chickens in the yard. Feeding chickens with suspected material has been suggested as a means of differentiating types.

B. tetani (*Clostridium tetani*) Carlo and Rattone caused tetanus in rabbit by inoculating pus from human case, 1884; Nicolaier produced tetanus by injecting animals with garden soil, 1885; Kitasato obtained pure cultures by incubating tetanus-containing material for 48 hours, then heating at 80°C. for one hour, thus killing nonsporing organisms, culturing anaerobically and producing tetanus in animals by inoculating the culture, 1885.—The tetanus bacillus is common in the faeces of horses and cattle, hence its frequency in cultivated soils. The horse is the most susceptible animal, next the guinea pig, then the mouse. The infection occurs in cattle and sheep. Dogs are much less susceptible. Fowls are practically immune.

The organism produces a soluble toxin which in man, after a period of several days' incubation, produces lockjaw, followed by tetanic convulsions of the muscles of the body. The usual period before symptoms occur is fifteen days. The shorter the period of incubation, the more probably fatal the disease. The infection occurs especially through skin wounds, and also from those of mucous membrane. The toxin is digested by the alimentary canal juices and infection by that atrium is improbable. While tetanus is, like diphtheria, a disease in which the bacilli are localized and do not spread, yet recently Richardson has obtained tetanus bacilli in pure cultures from the tributary lymphatic glands of a "rusty nail" wound of foot. The cultures inoculated into root of tail of a white rat caused the rat's death in forty-eight hours with typical "seal gait" attitude of tetanus in rats.

Morphological and cultural characteristics.—It is a long slender bacillus ($4 \times 0.4\mu$), slightly motile and forming a large (1.3μ) drumstick spore. Certain other anaerobes, as *B. tertius*, etc. form similar terminal spores so that a diagnosis of tetanus cannot be made by this finding alone. It is Gram-positive, but degenerating organisms in old cultures may lose this characteristic.

The spores are hard to find in material from tetanus wounds but readily develop in cultures at 37°C. after 2 or 3 days. It liquefies gelatin very slowly. In stab-culture in glucose agar it shows an "inverted pine-tree" growth. Colonies on agar plates show as fleecy clouds and microscopically as felted filaments. Milk is not coagulated.

Theobald Smith recommends growing it in fermentation tubes containing ordinary bouillon, but to which a piece of the liver or spleen of a rabbit or guinea pig has

been introduced at the junction of the closed arm and the open bulb. By this method spores develop rapidly in from twenty-four to thirty-six hours. Sporulation is most rapid at 37°C. As there is always liability to postmortem invasion of viscera by ordinary saprophytes, Smith recommends that great care be taken not to handle the animal roughly in chloroforming and in pinching off pieces of the organ at autopsy. The animal must be healthy, and the tubes to which the piece of tissue is added must be proven sterile by incubation. Smith calls attention to the uncertainty of the temperature at which tetanus spores are killed; he shows that with some a temperature only possible with an autoclave is required. In view of the danger of tetanus, it is advisable carefully to autoclave all materials concerned with the preparation of bacterial vaccines.

Tetanus types.—Tulloch made a report on the tetanus problem which brings out many practical points. He has established three types of tetanus bacilli by immunological reactions, I, II and III. The mortality from type I is less than from the other two types. There does not seem to be any specificity in the toxin so that an antitoxin made from type I toxin-antigen will protect against the toxins of the other types. In the production of the disease the devitalizing action on the tissues by other organisms, especially the Welch bacillus, is an important factor. Even the tetanus toxin seems to have little devitalizing effect. This would explain the failure of Francis to infect monkeys with vaccine virus grossly contaminated with tetanus. The great value of excision of the wound area over methods of dressing with antiseptics has been brought out (Debridement).

Tetanus and symbiosis.—*B. tetani* seems to grow better in symbiosis with aerobes; hence a lacerated dirty wound, or a neglected umbilical cord, each of which may be contaminated with various organisms and be difficult of sterilization, offers a favorable soil. The importance of the presence of ordinary pus cocci in a tetanus wound may be that the activity of the leukocytes in phagocytizing them allows the tetanus bacillus to escape phagocytosis. This would also explain the importance of necrotic tissue in a lacerated wound—the phagocytes taking this up instead of tetanus bacilli.

The toxin.—The tetanus bacillus, like *B. diphtheriae*, produces a soluble toxin. It is one of the most powerful poisons known, it being estimated that 0.0002 Gm. is fatal for man, and that it is twenty times as poisonous as dried cobra venom. There are in fact two toxins, tetanospasmin and tetanolysin, the former being the important one. That the disease is due to toxin is shown not only experimentally, but also by the fact that, if spores are carefully freed of all toxin by washing, and then introduced, they do not cause tetanus—the polymorphonuclears engulfing them.

Tetanus antitoxin.—The antitoxin is produced by injecting horses with increasing doses of tetanus toxin, following a preliminary dose of 5000 antitoxin units. An important point is that a horse used for the production of diphtheria antitoxin may become infected with tetanus and his blood contain enough tetanus toxin to kill. A number of children in St. Louis died of tetanus as the result of such an accident.

Rosenau has established an antitoxin unit for tetanus which has the power of neutralizing 1000 minimal lethal doses. Practically, it is ten times the least quantity of antitetanic serum necessary to protect the life of a 350-gram guinea pig from a test dose of tetanus toxin furnished by the Hygienic Laboratory. (The necessity of some definite unit is apparent since tests have shown that serum stated to contain 6,000,000 units per cc. had a value of only 90 of the official American units.)

Consequently it is a unit ten times as neutralizing as the diphtheria antitoxin one. The antitoxin of tetanus is less efficient therapeutically than that of diphtheria for the following reasons:

1. There is about three times as great affinity *in vitro* between diphtheria toxin and antitoxin as is the case with tetanus.

2. The tetanus toxin has greater affinity for nerve cells than for antitoxin.

3. Treatment with antitoxin is successful after symptoms of diphtheria appear. With tetanus it is almost hopeless after the disease shows itself. Hence the importance of the early bacteriological examination of material from a suspicious wound (rusty nail).

4. The tetanus toxin ascends by way of the axis cylinder, and the antitoxin being in the circulating fluids cannot reach it; whereas with diphtheria both toxin and antitoxin are in the circulation. Diphtheria also selects the cells of parenchymatous and lymphatic organs which are more tolerant of injury than the nerve cells.

Laboratory diagnosis.—In examining for tetanus, scrape out the granulation tissue or foreign material from the suspected wound with a sterile Volkmann spoon and insert into a pocket made with scissors

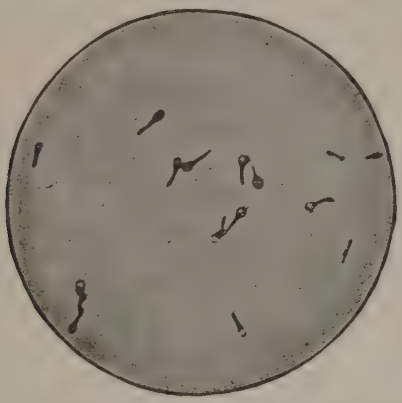


FIG. 30.—Tetanus bacilli showing end spores. (*Kolle and Wassermann.*)

in the subcutaneous tissues of the thigh of a guinea pig. Animal inoculation is the practical method. One may also put some of the suspected material in a Löffler's serum tube. In the incubator the luxuriant growth of the cocci and other aerobes enables the tetanus bacillus to develop. From day to day smell the culture, and if an odor that is penetrating, sour and foul develops, it is suspicious. The nondevelopment of a foul odor is against tetanus. Also make smears from the material and examine for drum-stick spores. If these are found, heat the material to 80°C. for one-half hour, to kill nonsporing

aerobes and facultative anaerobes, and then inoculate a deep glucose agar tube and cultivate by Wright's method. The fusiform lateral outgrowth about the middle of the stab is characteristic.

A more rapid method is to draw up the material, provided it be pus (tissue scrapings may be emulsified in sterile salt solution) into a capillary bulb pipette. Then seal off the end and heat the pipette and its contents in a water bath at 80°C. for fifteen minutes. Next break off the sealed tip and stick the pipette into a deep tube of glucose agar. When the point reaches the bottom, withdraw the pipette, forcing out the material along the line of the stab. Cover the surface of the agar with sterile liquid petrolatum and incubate. Better anaerobic conditions obtain where the Buchner or Wright method is employed.

Bacillus tetani produces no gas. Material for examinations is best obtained with a bulb pipette (containing a little sterile salt solution) which is plunged into the agar and the salt solution forced out and drawn in where a proper growth is noted.

Spores form in thirty-six to forty-eight hours. In injecting test animals it is advisable to divide the material to be injected into two portions; one animal is injected with the material alone, the second animal with tetanus antitoxin at the same time the material is injected. Only the first animal dies with tetanic symptoms.

Kenneth Taylor states that the search for tetanus bacilli in a wound is usually an unprofitable procedure and even if on rare occasions they can be identified their presence is of little significance.

Serum treatment.—The dose of tetanus antitoxin as a prophylactic is 1500 units; as a curative agent, 5000 to 20,000 units, the total dosage aggregating 100,000 units or more. Taylor notes the inefficiency of serum, however, and in whatever dose given, as is also true of carbolic acid and magnesium sulphate, when symptoms have set in. The mortality of cases developing within ten days is 80%. As the immunity of a prophylactic injection lasts only about ten days, subsequent operations on an infected area may start up acute tetanus. For this reason a prophylactic injection of about 1500 units should be given such cases prior to operation. Anaphylactic reactions following these secondary operations are rare and these can largely be obviated by subcutaneous injection of desensitizing doses of 0.5 cc. followed in three hours by the full dose.

Serum sickness showing oedema, urticaria, joint pains, vomiting and malaise may occur in from three to eight days after this injection. It usually lasts two or three days and the prognosis is good.

Chronic tetanus.—Taylor notes that chronic tetanus is not uncommon. The muscles about the wound may show clonic or tonic contractions, but trismus is frequently absent. A high pulse rate and no fever are rather characteristic. Cases may occur several months after operation or injury, especially with low-grade bone infections.

Bacillus welchii (*Clostridium welchii*) Welch, 1891.—This organism, commonly termed the "gas bacillus," was first isolated from gas gangrene cases and received the name *Bacillus aerogenes capsulatus*. Fraenkel gave the name *B. phlegmonis emphysematosae* to the same

organism which he also obtained from similar lesions. The name *B. perfringens* is used by the French. This is the cause of foamy liver and during the World War it was the most common cause of gas gangrene (75%), although nearly always associated with other organisms, especially *B. sporogenes* and certain aerobes. Apparently only certain very virulent strains of the gas bacillus can produce gas gangrene when present alone and then only when much necrotized tissue is present in the wound. It is this requirement which led to the development of the surgical procedure of "debridement."

FIG. 31.—*B. welchii* agar culture showing gas formation. (*Mac Neal.*)

Morphology and cultural characteristics.—The gas bacillus is a large ($5 \times 1.3\mu$) nonmotile Gram-positive anaerobe with square-cut ends. It forms oval, centrally placed spores, which but slightly distend the rod. These spores are not formed in the wound and it is difficult to bring about their development in ordinary culture media. Protein media, such as blood serum or egg, are the most favorable for spore formation. Spore formation is inhibited by the presence of sugar in media. The bacillus shows a capsule in smears from animal tissues or fluids. Some strains exhibit haemolytic power when grown on blood agar. Cooked meat media are in general best for culturing but the most characteristic cultural peculiarity is seen when the organism is grown in litmus milk, under anaerobic conditions. The tube of milk is inoculated with the material, heated to 80°C., and then incubated anaerobically for 18 hours. The optimum temperature for growth is 38°C. There occurs coagulation with disruption of portions of the coagulum into shreds, colored pink by acid formation and plastered against the sides of the tube. The gas-riddled mass of coagulum remaining floats in a clear whey which has the odor of butyric acid. To obtain pure cultures, inoculate 3 or 4 cc. of the whey into the ear vein of a rabbit. After 5 minutes kill the rabbit and place the carcass in the incubator for 6 to 8 hours. The body should become distended

with gas and the organism should be obtainable from the foamy liver or the heart blood (Welch-Nuttall test).

At the American Ambulance a sterile cotton throat applicator was used to obtain the discharge from a wound. Each swab is sent to the laboratory in a tube. A glucose agar tube is boiled for ten minutes, then quickly cooled to 42°C. The swab is then introduced into the melted agar and well rubbed up in it. This agar is then quickly solidified in ice water and put in the incubator. For quick diagnosis the swab, coated with agar, is transferred to the tube from which it was taken and this latter is placed in a larger tube containing pyrogallic acid and sodium hydroxide (anaerobic culturing). In four or five hours a smear can be made from this swab and stained by Gram to note presence of the gas bacillus.

Occurrence.—The gas bacillus is common in fertilized soils and in the faeces of man and other animals. Cultures from the clothing of men in the trenches almost always showed the Welch bacillus and less frequently the tetanus bacillus. Streptococci also were rather frequent. When a shell wound occurs we almost invariably have a gas bacillus infection, which during the first few days gives rise to a foul-smelling reddish-brown discharge. Smears from gas gangrene wounds, showing such discharge, have chiefly the gas bacillus and streptococci. In the second week the pus becomes more purulent and the gas bacillus is infrequent, while streptococci, staphylococci and coliform bacilli are abundant. Glucose agar to which about 0.1 cc. of blood has been added makes a very favorable medium for the gas bacillus. It also grows well on milk or blood serum. Fleming prefers neutral-red egg medium for its culturing. Cultures of the gas bacillus from gas gangrene discharges when injected subcutaneously into guinea pigs kill within twenty-four hours causing emphysematous swelling; death, however, being probably caused by other organisms. Chlorinated solutions seem to be more efficient against the infection than the formerly recommended hydrogen dioxide.

Pathogenic effect.—In studying the pathogenic effect of the gas bacillus, Taylor considered the gas as of little toxic importance. The mechanical effect of pressure produced is the most important part of the infection, causing death of tissues from pressure on blood vessels. It also causes fragmentation of muscle tissue and scattering of the infection. In treatment provision must be made for escape of gas.

Bull and Pritchett insist on the importance of a soluble (exotoxin) which produces both haemolysis and necrosis. By injecting rabbits with this soluble toxin they have produced an antitoxin. McGlennan has used this antitoxin (about 7500 units intravenously) in three cases of gas gangrene infection with two recoveries. It is a fact that Dakin's solution will destroy the toxin of the Welch bacillus.

Kendall has called attention to the importance of this organism in a certain proportion of cases of summer diarrhoea of infants. (See under chapter on Faeces, p. 611.)

Other Anaerobes of War Wounds. Bacillus sporogenes. (*Clostridium sporogenes*).—This organism differs from the gas bacillus and the *Vibrion septique* in being actively proteolytic whereas they act on carbohydrates rather than on proteins. Next to the gas bacillus, it was the organism most frequently encountered in war wounds and was regarded as the main cause of their foul odor. Like the gas bacillus, it is often found in human or animal faeces and in fertilized soils.

It is a bacillus with rounded ends ($5\times0.8\mu$), actively motile and Gram-positive; it liquefies gelatin and digests blood serum. It does not seem to be pathogenic but appears to exalt the virulence of the gas bacillus.

It is a common contaminant of other anaerobic cultures, and since its spores have great resistant power it is hard to separate it from organisms we desire to get in pure culture. Kendall considers Barber's single cell technique as valuable in effecting separation. Metchnikoff's organism, the Reading bacillus, *Bacillus XI* and—by some—Koch's organism of malignant oedema are regarded as identical with *B. sporogenes*.

Bacillus histolyticus. (*Clostridium histolyticum*).—This organism, like *B. sporogenes*, has marked proteolytic power, so that the intramuscular injection of cultures into guinea pigs may bring about digestion of the muscles down to the bone within 15 hours—without, however, having much effect on the health of the animal.

It is a motile bacillus, about 4 by 0.6μ, and forms oval spores which tend to be excentric. Cooked meat media as well as blood serum are rapidly digested.

Bacillus oedematiens (*Clostridium oedematiens*).—This bacillus is quite large ($6\times1\mu$) and is usually stated to be nonmotile, although some authorities report young cultures as motile. It is more strictly anaerobic than the other pathogenic anaerobes. It liquefies gelatin. The spores, which form readily, are large (1.5μ) and located excentrically. It produces a soluble toxin against which an antitoxin has been produced. Injection of the toxin (0.05 cc.) kills guinea pigs in one or two days.

This organism was a rather common one in the war wounds. Injected into the muscular tissues of a guinea pig it produces a gelatinous necrosis with only slight gas formation.

Bacillus fallax (*Clostridium fallax*).—This bacillus was a rather rare anaerobe of war wounds. It is smaller than the gas bacillus and has slight motility.

It produces a slight amount of gas but does not liquefy gelatin. Its spores, which are central or excentric, are not readily formed in culture media.

It produces a soluble toxin which gives rise to oedema. It is not a very pathogenic organism.

Bacillus tertius (*Clostridium tertium*).—This is a bacillus with rounded ends, averaging 5 by 0.5μ in size. It has sluggish motility. The spores are strictly terminal. It does not liquefy gelatin and does not digest blood serum. It coagulates milk with slight gas formation. It has no pathogenic effect on laboratory animals but may cause gas formation in wounds.

CHAPTER VII

STUDY AND IDENTIFICATION OF BACTERIA—MYCOBACTERIA, CORYNEBACTERIA AND PFEIFFERELLA. KEY AND NOTES

Key for Bacilli.—Having branching characteristics; showing parallelism, branching, curving forms, V-shapes, clubbing at ends, segmental staining, etc.

Acid-fast.—Mycobacterium. { Cultures more or less wrinkled and dry. / More like moulds.

I. Grow rapidly on ordinary media at room temperature.
   (1) Mycobacterium phlei. (Timothy grass bacillus of Moeller.)
   (2) Mycobacterium butyricum. (Butter bacilli as reported by (a) Rabinowitsch and (b) Petri.)

II. Grow only at body temperature. Scanty growth or none at all on ordinary media. Media of preference are: (a) Solidified blood serum, (b) glycerin agar, (c) glycerin potato and (d) egg media.
   A. Cultures fairly moist, luxuriant, and flat. Opt. temp. 43°C.
      (1) Mycobacterium avium. (Bacillus of avian tuberculosis.)
   B. (a) Cultures scanty, wrinkled and dry. Appear in ten to fourteen days. Opt. temp. 38°C. Bacilli longer (2.5μ) narrower, more curved, more regular in outline and staining than are bovine; vacuolation more marked. Smear from organs of inoculated guinea pig shows few bacilli. Less virulent for rabbits.
      (1) Mycobacterium tuberculosis (hominis). (Bacillus of human tuberculosis.)
   (b) Cultures as above, but even more scanty. Bacilli shorter (1.5μ), thicker, less vacuolated. Smear from organs of guinea pig shows many bacilli.
      (1) Mycobacterium tuberculosis (bovis). (Bovine tubercle bacilli.)
   C. Very difficult to cultivate (Czaplewski).
      (1) Mycobacterium smegmatis. (Smegma bacilli for various animals.)

III. Noncultivable by ordinary methods. Clegg cultivated in symbiosis with amoebae what he believed was *M. leprae*. Duval cultivated an acid-fast bacillus on N.N.N. medium containing 1% glycerin. Bayon cultivated on placental juice glycerin agar a slightly acid-fast diphtheroid which changed to acid-fast in peritoneum of mouse. Bayon's organism thought to be similar to Kedrowsky's diphtheroid of leprosy. Present view is that *M. leprae* is noncultivable.

(1) Mycobacterium leprae. (*B. leprae.*) Found chiefly in nasal mucus and in juice from lepra tubercles. Less often in nerve leprosy.

(2) Bacillus of rat leprosy. Indistinguishable from *M. leprae* except by inoculation into young rats.

Nonacid-fast.—Corynebacterium and Pfeifferella. { Colonies more flat and moist. / Like other bacteria.

I. Gram-positive: Corynebacterium.

(1) Corynebacterium pseudodiphthericum. (*B. hoffmanni.*) Very luxuriant growth on ordinary media. Colonies often yellow to brownish. Short, thick and stain uniformly.

(2) Corynebacterium diphtheriae. Moderate growth on ordinary media. Best media are blood serum (Löffler's) or glycerin agar. Has metachromatic granules at poles.

(3) Corynebacterium xerosis. Scanty and slow growth on nutrient media.

II. Gram-negative: Pfeifferella.

(1) Slender, poorly staining rods, forming threads with a tendency toward branching. Do not ferment carbohydrates. Characteristic honey-like growth on potato. Pfeifferella mallei (Glanders bacillus). Cause of glanders, affecting horses, man, sheep, and goats.

Note: B. whitmori the cause of melioidosis a glanders-like disease in man and rodents occurring in Burmah and the Malay States is serologically almost identical with *P. mallei*, but differs from it by reason of its motility and character of its growth on laboratory media.

The Group of Acid-fast Branching Bacilli

There is a large and ever-increasing number of organisms which have the same staining reactions as the tubercle bacilli.

These nonpathogenic acid-fast bacilli are of greatest importance by reason of their possible confusion with the true tubercle bacilli. Their colonies correspond more or less with different types of tubercle bacilli colonies, being either dry and wrinkled like human, or moist and irregularly flat as avian. Eventually the moist colonies become dry and wrinkled. They have been isolated from butter and milk; from grasses, especially in timothy grass infusion; from various excretions of animals as dung, urine, etc., and from the skin, nasal mucus, cerumen and tonsillar exudate of man, where they occur normally.

It is important to remember that such organisms have very rarely been reported also from pulmonary lesions, and when present they have been considered as probably causative.

These nonpathogens differ from the tubercle bacillus in five important essentials:

1. Grow readily on any media.
2. Showing more or less abundant growth, or colonies, in twenty-four hours.
3. Having no pathogenic power for guinea pigs when inoculated subcutaneously.

4. Not requiring body temperature for development, but growing at room temperature or a little above.

5. Being morphologically shorter and thicker.

Many of these organisms if injected intraperitoneally into guinea pigs will produce a peritonitis with false membrane. Some also produce granulation tissue nodules which may be confused with true tubercles. When injected intravenously localization is renal—very rarely pulmonary. Lesions tend to suppurate rather than caseate.

Tuberculosis in the guinea pig.—Owing to the similarity in appearance and staining reaction among these several organisms, animal inoculation must frequently be resorted to, so that it is well to study the lesions in experimental tuberculosis in the guinea pig. Injected subcutaneously, on either or both sides of the posterior abdomen with the needle pointing toward the inguinal glands, we may have caseation and ulceration at the site of inoculation. The glands in relation enlarge and caseate. Smears from these show tubercle bacilli. The marked and characteristic change is the enormous enlargement of the spleen, which is studded with grayish and yellow tubercles. Smears and cultures from the spleen show tubercle bacilli. The death of the guinea pig usually occurs in about two months. The lesions may be looked for at three to five weeks.

Notwithstanding the prevalence of acid-fast organisms, the present view is that the finding in the sputum of organisms having the appearance and staining characteristics of tubercle bacilli has practically as great value as it had before we knew of these various acid-fast bacteria.

Bacillus smegmatis (*Mycobacterium smegmatis*).—This acid-fast organism is of importance, chiefly from the fact that such bacilli may be found in urine and be reported as tubercle bacilli.

They show a greater tendency to appear in clumps, thus resembling lepra bacilli, but may have the appearance of typical tubercle bacilli. It is usually stated that they decolorize easily in alcohol but I have found smegma organisms in urine as resistant to acid alcohol as tubercle bacilli. Such organisms are found in preputial and vulvar secretions, and such location accounts for their presence in urine. The Lustgarten bacillus, reported in 1884 as the cause of syphilis, was probably a smegma bacillus. It is very difficult to obtain cultures (blood or serum media necessary). For differentiation always inoculate a guinea pig for which, as for man, the organism is nonpathogenic. Some believe that investigators who have reported the successful cultivation of lepra bacilli isolated from nasal mucus were working with smegma bacilli.

Bacillus tuberculosis (*Mycobacterium tuberculosis* [*hominis*]) Koch, 1882.—This is a rather long, narrow rod, $3 \times 0.3\mu$. In the human type it tends to show a beaded appearance, this not being due to spores, however. In the bovine type the staining is more solid, the organism shorter and thicker, and shows even a more scanty growth than the human tubercle bacillus.

Frequency of tuberculosis.—This is the most common of human diseases, probably 10% of all deaths being due to tuberculosis. The death rate is decreasing in the United States but was temporarily doubled, during the war years in Europe, due to the deprivations of war. Part of the present low mortality is due to removal of many infectious cases by the influenza epidemic of 1918. That epidemic may have thus helped to stamp out foci of infection in the various communities, as well as removed the weak who were potential candidates of tuberculous infection. The incidence, as shown by autopsy findings, increases with age, being rare under one year, more frequent after the fifth year and for those between twenty and thirty most common. There is a low mortality between five and fifteen years of age, and after forty. Examinations of very large groups of people indicate that 1% of the population in the United States has active tuberculosis and another 1% has arrested yet recognizable tuberculosis. Statistics (Beitzke) show tuberculous lesions in 58% of adults at autopsy—Naegli's figures were about 90%.

Bovine tuberculosis in man.—It has been established that many of the tuberculous affections of man, especially those of the skin, bone, and mesenteric glands, are of the bovine type while, as a rule, pulmonary and laryngeal lesions are of the human type. Experiments by various commissions in different countries have shown that human and bovine types are very closely related and that not only may a bovine strain affect man, but that human tubercle bacilli may infect young calves. As bacilli of the bovine type have frequently been reported in intestinal and mesenteric tuberculosis of children, the importance of sterilizing cow's milk is apparent.

Koch at one time considered human infection from bovine sources as of very rare occurrence and by his claims of the unimportance of milk from cows with tuberculosis as a source of human infection (1901) brought about intensive study of this problem. Later Koch modified his views.

Although Kossel has found only two cases of bovine infection in 709 cases of pulmonary tuberculosis yet for the other clinical types the findings are different. Leaving out of consideration the frequency of infections with bovine strains in children, statistics have shown that in adults about 4% of cervical adenitis, 22% of tabes mesenterica

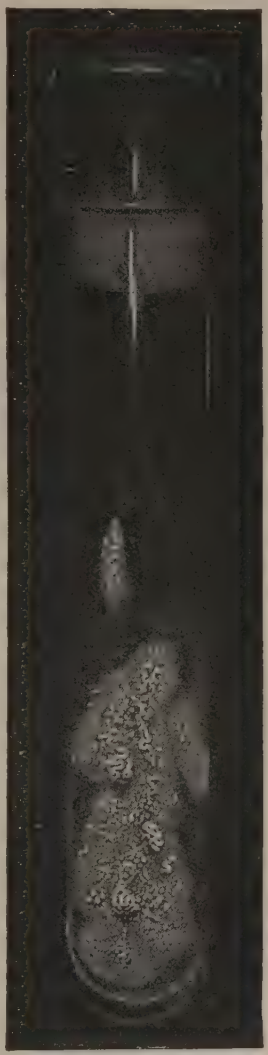


FIG. 32.—*Bacillus tuberculosis;* glycerin agar-agar culture, several months old. (*Curtis.*)

and 3.5% of bone and joint tuberculosis are due to bovine strains of the tubercle bacillus.

Park and Krumwiede, in a study of more than 1000 cases, found about 10% due to bovine tuberculosis. Of 686 adult cases only 1.3% showed bovine strains while 352 cases under sixteen showed approximately 25% of bovine infection. Of 592 cases of pulmonary tuberculosis, in children and adults, not a single case could surely be regarded as bovine.

Atrium of infection.—A subject of great moment is that of the atrium of infection in tuberculosis. While 75% or more of human cases show apparently primary infection of the respiratory tract, yet we now have views that the disease is rarely acquired by the aspiration of contaminated dust, as Cornet believed, or by Flügge's spray method of infection from droplets of sputum expelled in coughing. The path from intestinal tract to thoracic duct and lungs is direct, so that tuberculous infection of bronchial glands may be by way of intestines. Some European statistics, using von Pirquet's method of diagnosis, have shown 90% of children under fourteen infected while similar American ones have shown about 50%. The prevailing idea is that we get our infection in childhood and show the disease as we approach adult life. With bovine infections we are sure that infection of man takes place almost exclusively by the alimentary tract. Brown, Petroff and Pasquera carried out experiments as to mode of infection. Material from dust of rooms occupied by patients with marked cough and tubercle bacilli-laden sputum when inoculated into guinea pigs failed to infect a single one of the 24 animals used. In another experiment 3 guinea pigs were placed in a box connected with a vacuum cleaning apparatus and exposed to similar dust passing through the box; they did not acquire infection.

Calmette is of the opinion that tubercle bacilli reaching tonsils or intestinal tract are phagocytized by leukocytes, which carry them to the lymphatics, and that these organisms may lie dormant in lymphatic glands for long periods. He regards direct lung infection as rare.

Following further study, the 1927 conviction of Baldwin, Petroff and Gardner is:

(a) Living bacilli are present in the dust and occasionally in nasal mucus of contacts with careless patients.

(b) Primary lung infections in infants result from direct inhalation of tubercle bacilli.

(c) Small numbers of tubercle bacilli are frequently taken into air passages as evidenced by living bacilli in tracheobronchial and other lymph nodes without signs of tubercle formation.

(d) Whether or not the disease develops depends on the numbers and vitality of tubercle bacilli taken into the body, and the frequency with which they are received.

Secondary infections.—It is certain that many of the symptoms usually noted in the tuberculous are due to secondary infections. Pettit, by careful blood cultures, obtained the *Pneumococcus* in 24 cases and the *Streptococcus* in 36 cases out of 130 cases studied. He used from 5 to 20 cc. of blood from a vein. Positive blood cultures were obtained in 68% of far-advanced cases, 45% of advanced cases and 16% of incipient cases.

Types.—The British Royal Commission in its final report of July, 1911, considered three types of *Bacillus tuberculosis.*

I. The bovine type, belonging to the natural tuberculosis of cattle.

II. The human type, the type more generally found in man.

III. The avian type, belonging to natural tuberculosis of fowls.

Bovine and human types.—Bacilli of the bovine type are shorter and thicker than the human ones and tend to be irregularly arranged, while the human ones are longer, slightly curved and tend to lie parallel.

The bovine type grows slowly on serum, and at the end of two or three weeks shows only a thin grayish uniform growth which is not wrinkled and not pigmented. The human type grows more rapidly and tends to become wrinkled and pigmented. Subcutaneous inoculation of 50 mg. of bovine culture into the neck of calves produces generalized tuberculosis. A similar injection of human organisms does not cause generalized tuberculosis but only an encapsulated local lesion.

Intravenous injection of 0.01 to 0.1 mg. of bovine culture into rabbits causes general miliary tuberculosis and death within five weeks. With human cultures in doses of 0.1 to 1.0 mg., similarly injected, the majority of rabbits live for three months.

Subcutaneous injection of 10 mg. bovine tubercle bacilli causes death in 28 to 101 days. Similar injection of human organisms in doses up to 100 mg. do not kill rabbits after periods of from 94 to 725 days. The duration of life in injected guinea pigs is longer with human than with bovine inoculations.

Subcutaneous injection of bovine strains into cats produces generalized tuberculosis while the cat is resistant to human strains thus given.

Avian type.—The avian type grows at 43°C. fairly luxuriantly, as a moist, more or less spreading culture. It grows much better on glycerinated agar than on serum. Morphologically it is like the human type, but shows less tendency to form compact masses. Very pleomorphic. Has been reported from sputum of man (doubtful).

Fowls may be infected by intravenous or subcutaneous injection, or as the result of feeding. After feeding, the lesions are chiefly of the alimentary tract; after injections, of spleen, liver and lungs. Avian cultures are more virulent for rabbits than human but less so than bovine. The mouse is the only animal besides the rabbit in which avian tubercle bacilli can cause a generalized tuberculosis. The conclusions are that there is no danger to man from avian sources.

Cold-blooded animal type.—Certain acid-fast organisms have been isolated from fish, frogs and snakes. These organisms grow much more rapidly than the other types (three to four days), and grow best at 20° to 24°C., growth ceasing at 36°. The colonies are round and moist. As a rule they do not produce tuberculin and, as they may be isolated from algae, may be considered as harmless saprophytes. However, Aronson has reported a strain from salt-water fish which is tuberculin producing and pathogenic for mice, pigeons and frogs, but non-pathogenic for guinea-pigs.

Culture media.—A good culture medium for primary cultures is blood serum or, better, a mixture of yolk of egg and glycerin agar. Petroff's medium and Dorsett's egg medium are to be highly recommended. In subcultures, either glycerin agar, glycerin potato, or glycerin bouillon makes good media.

On Besredka's medium in the course of two or three weeks we have a thick membranous growth. It is stated that after a growth of from four to six weeks human strains show a dry scale-like growth which is easily detached from the sides of the flask, while bovine cultures show a sort of mucoid growth adhering to the walls.

In inoculating solid media from tuberculous material, as, say, from a tuberculous gland or, more practically, from the spleen of a guinea pig, the material must be thoroughly disintegrated or rubbed on the medium so that individual bacilli may rest on its surface. In growing in flasks in glycerin bouillon a surface growth is desired. The cylindrical flask of Koch gives a better support to the pellicle than an Erlenmeyer one. In inoculating, a scale of such a surface growth, or a grain from the growth on a slant, should be deposited on the surface of the glycerin bouillon in the flask. In cultivating from sputum use Petroff's medium.

Immunity.—The fact that healed tubercles are found in great frequency at the autopsy of those who have never given evidence of the disease shows the existence of a resistance to tuberculosis. Attempts to produce active immunity with tuberculins or living or dead organisms have not met with success. The same may be said of methods of passive immunization, as with that of Maragliano or that of Marmorek. During the late war it was noted that Senegalese troops had no resistance to tuberculosis and the disease in them was similar to that observed in a young child or a guinea pig. The disease started with enlargement of the glands at the root of the lungs and, after a glandular stage of one or two months, a generalized tuberculosis with caseous pneumonia, or miliary tuberculosis, set in, death almost invariably occurring.

Toxic products.—Inasmuch as the filtrate from cultures has little toxic effect, the poison is assumed to be intracellular.

Tuberculius.—Koch's "Old Tuberculin," which was simply a concentrated 5% glycerin bouillon culture, is now principally used in diagnosis. It was prepared as follows: After four to six weeks the surface growth begins to sink to the bottom of the flask. This fully developed culture is evaporated over a water bath at 80°C. to one-tenth the original volume. It is then filtered, the final product containing about 40% of glycerin.

Koch's "New Tuberculin" or "T.R." was introduced in 1897. In this, virulent bacilli are dried *in vacuo* and ground up until stained smears fail to show intact bacilli. One gram of such material is triturated with 100 cc. water and centrifugalized. The supernatant fluid is removed and is designated "T.O." The residue is then dried, triturated in water and centrifugalized. Subsequent trituration and centrifugalization, preserving each time the supernatant suspension, give the new tuberculin. Not more than 100 cc. of water should be used for the treatment of the residue. It has been found at times to contain virulent tubercle bacilli.

Koch's "bazillenemulsion" was later introduced by Koch (1901). This is simply a suspension of ground-up bacilli in 50% glycerin solution. It really is "T.O." and "T.R." combined and contains 5 mg. of bacillary substance in 1 cc.

Another preparation is the bouillon filtrate of Denys. This is the unheated Chamberland filtrate of broth cultures of human tubercle bacilli. It contains 0.25% phenol. It is the same as unconcentrated old tuberculin and, not being heated, is supposed to contain in addition important thermolabile products.

In the use of T.R. and bazillenemulsion, Sir A. Wright recommends doses of $\frac{1}{4000}$ mg., and he rarely goes beyond $\frac{1}{1000}$ mg. in treatment. These products come in 1-cc. bottles containing 5 mg. of bacillary material. It is convenient to remove 0.2 cc. containing 1 mg. Add this to 10 cc. of glycerin salt solution with 0.25% of phenol. Each cc. contains $\frac{1}{10}$ mg. One cc. of this stock solution added to 99 cc. of salt solution, with 0.25% of phenol, would give a working solution, each cc. of which would contain $\frac{1}{1000}$ mg. of tuberculin.

Diagnosis.—For diagnostic tuberculin reactions we have the following:

1. Subcutaneous injection of $\frac{1}{5}$ mg. of old tuberculin. If no reaction occurs in four or five days we may increase to 1 to 5 mg.

Positive reactions show (*a*) constitutional symptoms of fever, malaise and possibly chill; (*b*) focal symptoms, as when a tuberculous gland, joint or skin involvement becomes active, and (*c*) local reaction as shown by the tenderness, induration or inflammation at the site of injection.

2. Variations in opsonic index following injection of a tuberculin.

3. Instillation into one eye of a drop of 0.5% or 1% solution of purified tuberculin. (Calmette.)

Reaction is shown by redness, especially of inner canthus, in twelve to twenty-four hours. A previous instillation may sensitize a nontuberculous case and a second application of the drop may give an erroneous diagnosis. This test should not be used in persons over fifty or when there is any disease of the eye to be used or for that matter of the other eye. For instance, in corneal opacities, due to tuberculous keratitis, a focal reaction would occur.

4. The cutaneous inoculation method (similar to ordinary vaccination methods). Scarify two small areas on the arm ($\frac{1}{10}$ inch in diameter) about 2 inches apart. Rub in one a drop of old tuberculin, in the other a drop of 25% tuberculin. As a control scarify a spot midway and to one side of the others and rub in 1 drop of 0.5% carbolic glycerin. The appearance of bright red papules in twenty-four hours indicates reaction (von Pirquet.)

It is now recommended to deposit a loopful of undiluted tuberculin on the skin and below that a loopful of saline. A linear incision with a sharp scalpel or glass needle is made through the saline and then through the drop of tuberculin trying not to draw blood. After two to five minutes the tuberculin is wiped off. No dressing is required. Reaction usually appears as induration or inflammatory areola or vesicles after twenty-four hours, but may be delayed forty-eight hours. This is the method of preference.

5. Intracutaneous inoculation of 1 drop of a 1–1000, 1–100 or 1–10 dilution of old tuberculin (Mantoux and Moussu).

Webb recommends hypodermic needle points which have been dipped in old tuberculin and then allowed to dry. A drop of water is placed on the skin and the needle points having been moistened in it are plunged through the skin and withdrawn with a twist. A definite lump shows a positive reaction.

6. Ointment tuberculin test. Rub in 50% ointment of tuberculin in lanolin. Reaction is shown by dermatitis with reddened papules in twenty-four to forty-eight hours (Moro).

7. Inoculation of bovine and human tuberculins to diagnose type of infection (Detre). Of questionable value.

Laboratory diagnosis.—Although we can usually make a diagnosis by staining sputum or other tuberculous material, results are more positive when a guinea pig is injected with the material.

It will be noted that a small indurated nodule appears in about ten days at the site of inoculation of the guinea pig. This softens and forms an abscess communicating with an ulcerated area, the tuberculous chancre. By squeezing this ulcerated area we may get a discharge which, when stained, shows tubercle bacilli.

Ebright injects the suspected material into the subcutaneous tissue of one side of the abdomen of 3 guinea pigs. At the end of one week an injection into the other side of the abdomen of one of the guinea pigs of 0.25 cc. tuberculin is given. Twenty-four hours later smears are made from the original site of inoculation and examined for tubercle bacilli. If negative this is repeated with a second guinea pig at the end of the second week and finally at the end of the third week with the third guinea pig.

Bloch's method is to damage the lymphatic glands in the inguinal region by squeezing the tissue between the fingers. Injections made there of tuberculous material show abundant tubercle bacilli in these damaged glands in ten to twelve days. See page 130 for tuberculosis in guinea pig.

Clough has noted the value of blood cultures on Petroff's medium in cases of miliary tuberculosis. Such findings are practically negative in other forms of tuberculosis (6.7%), but in miliary tuberculosis the findings were 66.6%.

Staining.—In staining it is better to use the Ziehl-Neelsen method, decolorizing with 3% hydrochloric acid in 95% alcohol. The alcohol, for all practical purposes, enables us to eliminate the smegma and similar bacilli, these being decolorized by such treatment.

Cooper adds 3 cc. of 10% sodium chloride to 100 cc. of Ziehl-Neelsen carbol fuchsin, which he keeps in incubator to prevent precipitation at room temperature. Slides are left in above solution (in incubator) overnight, then container with slides is transferred to ice-box for 20–30 minutes (until stain precipitates). Wash, decolorize and counterstain one minute with 1% brilliant green in 1 to 10,000 sodium

hydroxide. The NaCl increases visibility of tubercle bacilli, so that this method is said to have increased the positive findings 10% at Trudeau Sanitarium.

There are two objections to the Gabbett method, where decolorizer and counterstain are combined: 1. We cannot judge of the degree of decolorization—we are working in the dark; and 2. the matter of elimination of smegma bacilli is impossible.

Pappenheim's method, in which decolorization and counterstaining are accomplished by corallin and methylene blue dissolved in alcohol, is the standard staining method for the differentiation of tubercle and smegma bacilli. As a matter of fact, when the question of tuberculosis of the genito-urinary tract is involved, a guinea pig should be inoculated with urinary sediment.

It must be remembered that in young cultures of tubercle bacilli many of the rods are nonacid-fast taking the blue of the counterstain, while older rods are acid-fast. This fact frequently causes one to suspect that the culture is contaminated.

Discussion has arisen as to the granules of Much. These are considered by Much as resistant forms while others consider them degeneration forms of tubercle bacilli. At any rate material containing only these Gram-positive granules and no acid-fast rods may when injected into animals give rise to tuberculosis and acid-fast bacilli.

The combination of the acid-fast and Gram-staining methods as recommended by Fontes is very satisfactory.

Bacillus leprae (*Mycobacterium leprae*) Hansen, 1874.—This is the cause of leprosy. In nodular leprosy the organism is readily and in the greatest abundance found in the juice of the tubercles of the skin, and secretions of ulcerations of nasal and pharyngeal mucosa; in nerve leprosy, however, the finding of bacilli is rare.

The earliest lesion is possibly a nasal ulcer at the junction of the bony and cartilaginous septum. Scrapings from this ulcer may give an early diagnosis.

In the skin the bacilli are chiefly found in the derma packed in the so-called lepra cells. The process is granulomatous but does not show the caseation of tuberculosis or the predominant plasma cells of syphilis. The bacilli are also found engulfed in the endothelial cells lining the lymphatics.

They are also found in the glands in relation to the superficial lesions. The bacilli are found in smaller numbers in the liver and spleen. In anaesthetic or nerve leprosy they are found in small numbers in the granuloma tissue which invades the interstitial connective tissue of the peripheral nerves, and, rarely, in the anaesthetic spots of nerve leprosy.

The leprosy bacilli are found in profusion in the granulomatous tissue of the corium and subcutaneous structures of the leprous nodules, chiefly within cells called "lepra cells" and also within endothelial and connective-tissue cells as well as lying free, packed in lymphatic channels, thus forming the so-called "globi."

Differentiation from B. tuberculosis.—The leprosy bacillus may be distinguished from the tubercle bacillus by the following points:

1. The presence ordinarily of huge numbers of bacilli often grouped in packets like a bundle of cigars tied together. It will be remembered that it is very difficult

to find even a single tubercle bacillus in a skin lesion. Leprosy bacilli form palisade groups but not chains.

2. The leprosy bacilli stain more solidly and granules, when present, are coarser and more widely separated than the fine granulations of the tubercle bacillus.

3. They do not stand decolorization quite as well as the tubercle bacillus. With 20% sulphuric acid in water they hold their color almost as well as tubercle bacilli, but with 3% HCl in alcohol they decolorize in about two hours as against twelve to twenty-four hours for the tubercle bacillus.

4. Leprosy bacilli have neither been surely cultivated nor surely inoculated with pathogenic results into guinea pigs or other experimental animals and it is by the negative results upon cultivating or animal inoculation that we have our surest method of differentiation from tubercle bacilli.

Leprosy bacilli are chiefly spread through the lymphatics and, with the exception of the corium, it is in the lymphatic glands that they are found in greatest abundance. In nodular leprosy, however, their occurrence in the blood stream during the febrile accessions is so constant that this route also may be of importance.

Cultivation experiments.—A great amount of work has been done in attempting to cultivate the leprosy bacillus.

In 1900 Kedrowsky, culturing material from 3 cases of leprosy, obtained diphtheroids from 2 and a streptothrix from 1. A rabbit was inoculated first intravenously and later intraperitoneally with this nonacid-fast streptothrix and when killed six months later showed peritoneal nodules from which both diphtheroids and acid-fast bacilli, but not a streptothrix, were recovered culturally. Injection of cultures of the acid-fast bacilli and diphtheroids into rabbits and mice produced nodules which, when cultured, showed acid-fast organisms or diphtheroids. In 1901, he cultivated a diphtheroid from a fourth case of leprosy.

Fraser and Fletcher working with Kedrowsky's culture produced peritoneal nodules with the killed as well as the living organism. They were able to produce the same results with *B. phlei.*

With emulsions of leprous nodules, rich in leprosy bacilli, they could not produce similar lesions in the experimental guinea pigs.

Rost obtained a culture on a salt-free medium from which he prepared his leprolin by a process similar to that used for old tuberculin. It was claimed that leprolin had marked curative power in leprosy. Williams and Rost have cultivated a streptothrix on a medium containing milk.

Clegg, by inoculating his medium with cultural amoebae, obtained growth of a diphtheroid organism, with acid fast tendencies, from the spleen pulp of lepers.

Duval, by using media containing amino acids, obtained by tryptic digestion, brought forward two organisms, one of which was a diphtheroid and grew luxuriantly while the other showed a slow scanty growth and was acid-fast.

Bayon, by using placental media, isolated an organism rather resembling that of Kedrowsky. These organisms alone responded to immunity tests when such were made by Bayon and they alone gave rise to tissue changes resembling those of leprosy when injected into animals.

Professor Deycke obtained a streptothrix-like growth from the granulomatous tissue of excised leprous nodules. The ethereal extract from this culture gave a neutral fat which he called nastin and which is the basis of a leprosy treatment.

After working for eighteen months with material from 32 nonulcerative cases of nodular leprosy, not only with media as recommended by Duval, Rost and Bayon, but with blood and serum culture media, both by aerobic and anaerobic procedures, Fraser has been unable, in a single instance, to obtain any evidence of growth from this wealth of leprosy material.

As evidence against the possibility of culturing the human leprosy bacillus, it may be stated that most of the experiments along this line with rat leprosy, a disease occurring naturally in rats and caused by an organism almost identical, as to lesions produced, with the leprosy bacillus, have been negative. Bayon, however, states that he has cultivated the bacillus of rat leprosy.

Transmission.—There is nothing definitely known as to the method of transmission of the disease. Young children are particularly liable to infection, intimate contact in childhood being an epidemiological factor of importance. The itch mite and *Demodex* have been suggested as transmitting agents, and there is more reason for suspecting them of being concerned in transmission than for suspecting lice, bedbugs, mosquitoes or house flies. Personal uncleanliness and overcrowding appear to favor infection.

Rat leprosy.—Recently a leprosy-like disease of rats has been reported in which there are two types: 1. A skin affection and 2. a glandular one. In this disease, acid-fast bacilli, alike in all respects to leprosy bacilli, have been found. Deane has obtained a diphtheroid-like organism in culture, which is nonacid-fast. This same finding has been obtained in cultures considered positive in human leprosy.

In rat leprosy it has been found that infection of other rats takes place as readily through slight abrasions of the skin as when material is injected subcutaneously. According to Uchida leprosy developed in about six months in young rats (*Rattus decumanus*) when inoculated. Experiments with white rats and mice were negative. Material from human lepers failed to infect rats.

The idea is that natural infection occurs by way of the skin and through the lymphatics. There is no evidence that insects play a part in transmission.

Rat leprosy prevails extensively in Europe, Asia and America. Although similar etiologically and pathologically there does not seem to be any connection between the disease in rat and in man, differing in this respect from human and rat plague.

Laboratory diagnosis.—The usual procedure is to scrape a spot or nodule with a scalpel until the epidermis has been gone through and then smear out the exuding serum on a slide and stain by the Ziehl-Neelsen acid-fast method or by Gram's stain. Twenty per cent. sulphuric acid is less apt to decolorize than the 3% acid alcohol, the leprosy bacilli being less resistant to acid alcohol decolorization than to that by aqueous acid solutions. There is a great variation in the resistance to decolorization of leprosy bacilli, those from one case holding their color almost as well as tubercle bacilli, while those from another case may decolorize very easily.

I am partial to Tschernogabow's technique. In this, one punctures the subepithelial granulomatous tissue with a capillary pipette, the end of which has been broken off by tapping the point in order to give a cutting point, and the serum which exudes is smeared out and stained. Greenbaum and Schamberg recommend the aspiration of material from nodules or glands with a small glass syringe containing a few drops of saline. After introduction of the needle, the fluid is injected and drawn in and out of the syringe several times to emulsify the bacilli-containing tissue.

Some prefer emulsifying a piece of the tissue and centrifuging and staining the sediment. The antiformin method of treating leprous tissue, as for tuberculous tissue, has been used.

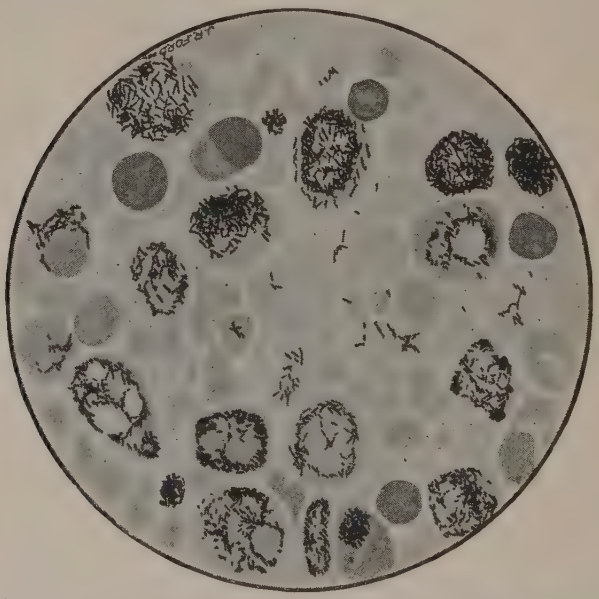


FIG. 33.—Section of spleen showing lepra cells and lepra bacilli. ×800. By permission from Manson's Tropical Diseases.

Many insist that the best method is to cut out small sections of the lesion, going well into normal tissue, and putting through paraffin and cutting thin sections and staining. Gram's method, counterstaining with Bismarck brown, gives beautiful preparations. The organisms are Gram-positive. For acid-fast staining, first stain with haematoxylin to obtain a histological background and then steam with carbol fuchsin, decolorize very briefly with acid alcohol, then put through absolute alcohol and xylol.

Smears from nasal mucus.—Of the greatest diagnostic value is the staining of the nasal mucus or scrapings from ulcerations on nasal septum for leprosy bacilli. These are often found in the characteristic

cigar package bundles or engulfed in lepra cells. A standard procedure is to give 60 grains of iodide of potash to cause a drug coryza, in the secretions of which leprosy bacilli may be found. However, one will have better success if the nasal secretion be obtained at a time when a natural coryza exists.

Thibault examined the nasal mucus, gland juice and blood of 30 lepers. He obtained leprosy bacilli in the nasal mucus of 20, in the gland puncture juice of 18, and in the blood of 7.

Hollman detected leprosy bacilli in the nasal mucus of 90% of 58 nodular cases, of 67% of 6 mixed leprosy and of 45% of anaesthetic cases, after making 329 examinations.

The blood in leprosy.—Leprosy bacilli are apt to be found in the blood of nodular cases, especially at the time of the febrile accessions. The blood is best taken in 5 or 10 cc. quantities into 1% sodium citrate in distilled water. After centrifuging, the sediment is treated with 10% antiformin, at 37°C. for one hour. Again centrifuging, and washing, the sediment is smeared out on a slide and stained. The bacilli are not apt to be found in the blood of cases of nerve leprosy.

It must not be forgotten that while the finding of leprosy bacilli is usually very easy in the nodules of nodular leprosy, search is a painstaking and discouraging procedure with the spots of nerve leprosy. Even the affected nerves, at autopsy, often fail to show bacilli. For nerve leprosy the examination of nasal mucus is of prime importance, but often results negatively.

The Wassermann reaction in leprosy.—There have been many reports of positive findings with the Wassermann test in cases of tubercular leprosy but such findings are by many considered doubtful. Butler, in the Philippines, has found that the lepers at his clinic gave no higher percentage of positive Wassermann reactions than did the nonleprous native patients. Sutherland and Mitra obtained 17 positive Wassermann reactions in 34 nodular cases, 16 positives in 52 anaesthetic cases and 8 positives in 14 cases of mixed leprosy. The sera of 12 children of leprous parents were negative. Kolmer has reported his complement fixation test and the Kahn test as giving negative results with sera from non-syphilitic lepers.

The X-ray has been utilized in the recognition of the very, early trophic changes in bone, showing the commencing absorption of phalanges.

Nonacid-fast Branching Bacilli

Bacillus diphtheriae (*Corynebacterium diphtheriae*) Klebs discovered, 1883; Löffler cultivated, 1884.—The diphtheria bacillus is found not only in the false membrane which is so characteristic of the disease, but may be found in abundance in the more or less abundant secretions

of nose and pharynx. In studying the epidemiology of diphtheria, especial attention must be given to the examination of nasal discharges.

Carriers.—With diphtheria carriers it is important to remember that the crypts of the tonsils may harbor the bacilli and thus protect them from the ordinary application of antiseptic agents. Goldberger found that the combination of throat and nose cultures gave much higher findings with carriers than either separately. The nose cultures gave more positives than the throat ones. He obtained only about 1% of positives in 4093 cases in Detroit, these figures being lower than those from other sources. It is interesting to note that 32% of these people showed pseudo-diphtheria bacilli.

Localization.—The throat and nose are the sites usually invaded by the diphtheria bacillus but wounds of the skin may show diphtheritic membrane. A form of tropical ulcer, designated Veld sore, common in various desert regions, has been shown in

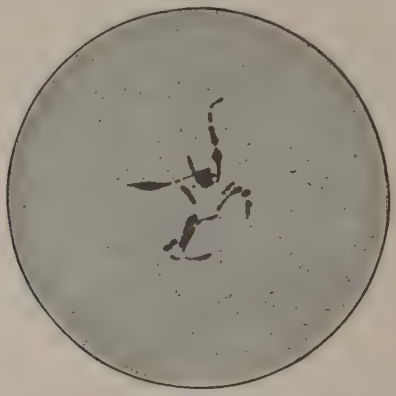


FIG. 34.—Diphtheria bacilli involution forms. (*Kolle and Wassermann.*)

certain cases to be connected with cutaneous diphtheria. Infections of the larynx and middle ear are not very rare. The mucous membrane of the vagina or the conjunctiva may also be infected.

The *B. diphtheriae* may be in pure culture, lying entangled in the fibrin meshes or contained within leukocytes in the membrane, or be associated with staphylococci, pneumococci, or especially streptococci. These latter may cause unfavorable complications as suppurative conditions about the neck. In fatal cases the diphtheria bacillus may be found in the lungs. Ordinarily, however, it remains entirely local and does not get into the circulation or viscera, the general symptoms being caused by its exotoxins.

Toxic products.—It produces soluble absorbable poisons which are designated *toxin* in the case of the one responsible for the acute intoxication, parenchymatous degeneration and death, and *toxone* for the poison which produces oedema at the site of inoculation and postdiphtheritic palsy. The injection of the soluble poisons alone without the bacilli produces the symptoms of the disease.

Morphology.—The bacilli tend to appear as slightly curved rods, showing varying irregularities in staining, as banding or beading, and in particular the presence at either end of small, deeply staining dots (metachromatic granules). It is nonmotile and does not form spores.

The granules may be seen in an eighteen-hour culture, but are more abundant after thirty-six hours. The granules are well brought out with Löffler's blue, but better with Neisser's method. In culture, the bacilli show swelling at one or both ends or clubbing. In secretions or in culture they show V-shapes or false branching and, what is most characteristic, the parallelism—four or five bacilli lying side by side like palisades. Being a Gram-positive organism while the majority of the other pathogenic bacilli are Gram-negative, it is of greatest importance to stain smears by

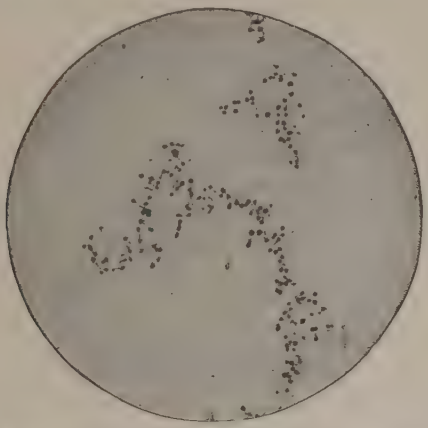


FIG. 35.—*B. diphtheriae* stained by Neisser's method. (*Mac Neal.*)

Gram's method. It is not so strongly tenacious of the gentian violet as are the cocci so decolorization should not be carried too far. According to their appearance when stained with alkaline methylene blue, Wesbrook divided diphtheria bacilli into solid, granular and barred types. The same organism may, however, show these variations with varying age of culture.

Cultural characteristics.—*B. diphtheriae* grows best at 38°C. and in a medium of about + 1 reaction. The best medium for growing it is Löffler's blood serum. It also grows well in milk which it does not clot or acidify.

An egg medium, made of the whole egg with glucose bouillon as described previously, is as suitable as Löffler's serum. Coagulated white of egg answers fairly well, as will a hard-boiled egg—the shell at one end being cracked and the white cut with a sterile knife. This smooth side is then inoculated and the egg placed cut side downward in a sherry glass. If an incubator is not at hand a tube may be

carried next the body in a pocket. The bacillus grows better on glycerin agar than on plain agar. On such plates they appear as small, coarsely granular colonies with a central dark area. In size the colonies resemble the streptococcus. On blood serum the colonies are larger—$\frac{1}{12}$ to $\frac{1}{8}$ inch in diameter.

The diphtheria bacillus grows luxuriantly on blood agar and on this medium shows a narrow zone of haemolysis around the colonies—narrower, however, than that of haemolytic streptococci. Diphtheroids do not produce haemolysis. In bouillon it tends to form a surface growth. It is at the surface that toxin production is most marked; hence in growing diphtheria for toxin formation we use Fernbach flasks which expose a large surface to the air. It is a marked acid-producer—glucose bouillon with a +1 reaction becoming +2.5 to +3 in thirty-six hours. If very little glucose is present the primary acidity is followed by alkalinity.

The Greenspon's medium (see p. 41) is said to be superior to Löffler's serum in that, through the action of the citric acid contained in the medium, the growth of many of the common mouth organisms is inhibited and the growth of the diphtheria bacillus is accelerated.

Toxin and the antitoxin unit.—The soluble poisonous products of the growth of the diphtheria bacillus, injected in increasing amounts, beginning with a nonlethal dose, are capable of provoking reactions on the part of an animal body which result in the production of neutralizing agents capable of completely abolishing the action of the poisons. The serum from the animal treated with the poisons—immunized, in other words—is the antitoxin and is of great value both in the prophylaxis and in the treatment of cases of diphtheria.

It is obvious that unless we have some recognized means of determining the power of the antitoxin in neutralizing poisons produced by the diphtheria bacillus we would have no satisfactory method of determining the dosage of the curative and prophylactic agent.

The strength or neutralizing power is usually indicated by the word "potency" and is determined by the number of antitoxic units a given antitoxin contains.

The antitoxic unit, like all other standards, is an arbitrarily fixed amount of antitoxin furnished by an official agency and used as a unit of measure and comparison, as are other standards.

In making tests to determine the potency of a sample of antitoxin it is necessary first to determine the test dose of toxin to be used against the standard antitoxin. This is accomplished by mixing varying doses of toxin with a constant dose of antitoxin of known strength (the standard unit) until a mixture is produced which will kill standard guinea pigs of about 250 grams in about four days.

Weight of guinea pig, grams	Dose of toxin, cubic centimeters	Dose of antitoxin	Death	Lesions
250	0.225	1 unit	5 da. 6 hrs.	Characteristic
250	0.230	1 unit	4 da. 4 hrs.	Characteristic
250	0.235	1 unit	3 da. 9 hrs.	Characteristic

Here the proper test dose of the toxin is obviously 0.23 cc., the larger and smaller doses being too high and too low respectively.

The quantity of toxin in this case, 0.23 cc., which will kill guinea pigs in this manner is known as the test dose or the L+ dose. This L+ dose in turn is mixed with varying doses of the antitoxin of unknown strength until the quantity of the unknown serum is ascertained which will just permit the standard guinea pig to survive about four days. This amount of course contains 1 unit of antitoxin and the number of units per cubic centimeter, or for any other volume of serum, can readily be computed.

Using the dose (0.23 cc.) of toxin as the L+ dose the following is an example of testing an unknown serum:

Weight of guinea pig, grams	Dose of toxin, cubic centimeters	Dose of antitoxin	Death	Lesions
250	0.23	(Control standard) 1 unit (Serum unknown)	4 da. 4 hrs.	Characteristic
250	0.23	0.01 cc.	2 da. 10 hrs.	Characteristic
250	0.23	0.0125 cc.	3 da. 22 hrs.	Characteristic
250	0.23	0.015 cc.	7 da. 6 hrs.	Characteristic

In this case the dose of 0.0125 cc. of unknown antitoxin protects for three days and twenty-two hours and may be regarded as containing one unit; hence, 80 units would be contained in 1 cc. of the serum.

A term sometimes used in this work is the "L o dose" which is just the amount of toxin required to accurately neutralize one unit of antitoxin without either acute or late symptoms developing in the guinea pig.

The strength of toxin for some purposes is designated by still another term—the minimum lethal dose (M.L.D.). It is employed

chiefly to indicate the amount of toxin (poisonous filtrate) which will kill standard guinea pigs in about four days, no antitoxin being used for neutralizing purposes. This dose of the poison is difficult to ascertain accurately, many animals being used for the purpose, and is usually ascertained only approximately for the purpose of determining the toxicity of the toxin for the immunization of horses in the commercial production of antitoxin.

Theoretically, according to the conception of Ehrlich, the standard unit of antitoxin combines with 200 units (M.L.D.-s) of a *pure* toxin.

The toxic products of the growth of the diphtheria bacillus, however, practically never consist of pure poison (toxin) alone, but contain varying amounts of other substances which have the power of combining with antitoxin but do not produce acute effects in animals. These other substances reduce the amount of pure toxin neutralized to one-half or less. Two of these toxic products are of sufficient importance to be briefly discussed in addition to the toxin.

Toxin is the product found in toxic filtrates of the diphtheria bacillus which is capable of causing acute death; this is not stable, decreasing gradually with age and becoming converted into *toxoid;* the latter is scarcely at all toxic.

Toxones are the agents which are capable of producing late manifestations in animals or man, chiefly nerve changes resulting in paralysis.

These three substances, *toxins*, *toxoids* and *toxones*, while differing much in action on the animal body, all have the capacity of combining with antitoxin. The three poisons differ in proportion in different poisonous filtrates (toxins) and the proportions differ in the same toxin at different times.

The standard unit of diphtheria antitoxin is distributed by official Government Laboratories, the Hygienic Laboratory of the Public Health Service at Washington being the agent which performs this function in the United States. This antitoxin in the form of dry powder is kept under conditions under which it undergoes practically no deterioration.

The toxin for standardization must be a seasoned product which has undergone the deterioration which is inevitable in this class of materials. In carrying out potency tests on antitoxins of unknown potency this seasoned toxin and appropriate dilutions of the antitoxin are mixed and incubated for a short period prior to injection into test animals.

Antitoxin.—In the preparation of antitoxin horses are employed, the method being to inject the bouillon filtrate or toxin subcutaneously at weekly intervals for a period of three or four months. When each cc. of the serum of the horse is found to contain about 250 to 500 antitoxin units the horse is bled from the jugular vein. Some sera contain as much as 1300 units in a cubic centimeter.

Methods of purifying and concentrating antitoxin are now employed by certain makers, the principle being that the antitoxin in the horse serum is precipitated with the globulins which come down on half saturation with ammonium sulphate. By thus excluding other proteids, the total amount of horse-serum proteid present with any given dosage of antitoxin is reduced, and the dangers of serum reaction correspondingly lessened.

As a curative measure, from 2500 to 5000 units should be injected. If the injection is delayed or the case very serious the dose should be 10,000 units.

As much as 30,000 units has been given in severe cases. In such cases intravenous injection is better than the subcutaneous one in the loose tissues of the loins. It is better to give the full dose in one injection. The prophylactic dose is 500 units. Joint pains and rashes may follow injection, as may, very rarely, sudden death. See p. 297 for "desensitization."

Schick reaction.—By the employment of this reaction we can understand why one child develops clinical diphtheria and another only shows the organism in the throat (laboratory diphtheria). We find that certain persons have sufficient amount of diphtheria antitoxin normally in the circulation to protect against the soluble toxin elaborated by the organisms localized in throat or nose. Such cases show either a minimal or negative reaction. Persons not having any antitoxin in the circulation show a positive reaction.

The test is performed as follows: With a small, sharp hypodermic needle we inject intradermally $\frac{1}{30}$ of a minimum lethal dose (M.L.D.) of diphtheria toxin as determined for a 250-gram guinea pig. The standardized toxin is so diluted with a 0.5% carbolic acid solution that 0.1 cc. contains $\frac{1}{30}$ of an M.L.D. A positive reaction shows within twenty-four hours, reaching its maximum intensity in two to three days, as a reddened area, about 1 inch in diameter with more or less induration. As a control it is best to inject an equal amount of the diluted toxin *heated* to 75°C. for one hour into the flexor surface of the other arm and if similar reactions occur with heated and unheated toxin to record it as a *pseudo-reaction*. The reaction persists for about a week, leaving a brownish pigmentation.

Positive reactions show that the patient has less than $\frac{1}{30}$ of a unit of antitoxin in 1 cc. of his blood serum and that he possesses no immunity to diphtheria. The following table shows the findings in New York City where Park and his coworkers have done a large amount of work in connection with the Schick test and subsequent

immunization. It is to be noted that the percentage of positive reactions is considerably higher among those living in the country. The infrequency of positive reactions during the first half year of life is due probably to an immunity inherited from the mother. That this immunity is lost to a large degree later on is shown by the figures for subsequent age periods. After five years of age natural immunity is slowly increasing.

AVERAGE SUSCEPTIBILITY OF VARIOUS AGES TO DIPHTHERIA (AS INDICATED BY THE POSITIVE SCHICK DIPHTHERIA-TOXIN SKIN TEST) IN NEW YORK CITY (AFTER PARK)

Age	Schick + (Susceptible)
Under 3 months	15 per cent.
3 to 6 months	30 per cent.
6 months to 1 year	60 per cent.
1 to 2 years	60 per cent.
2 to 3 years	60 per cent.
3 to 5 years	40 per cent.
5 to 10 years	35 per cent.
10 to 20 years	25 per cent.
20 to 40 years	18 per cent.
Over 40 years	12 per cent.

This test is of great value as showing the cases needing prophylactic injections of antitoxin. Furthermore nurses showing a positive reaction should not take care of diphtheria patients. Carriers of true diphtheria usually show a negative reaction as contrasted with pseudo-diphtheria carriers in whom the same incidence is observed as in any similar social group.

The test is also of value as indicating duration and degree of immunity following antitoxin injections. Investigations made with regard to the immunity conferred have shown that intravenous injections are the most efficient, next the intramuscular and least efficient the subcutaneous.

Immunization against diphtheria.—It is well known that the prophylactic injection of diphtheria antitoxin gives but a short immunity—not more than ten days. Where an epidemic is present or possibly in connection with persons not showing immunity by the Schick test, one can give a more lasting (active) immunity by treating the individual with the diphtheria toxin-antitoxin mixture. This method is especially of value in case of children (schools, infant asylums). A slightly underneutralized mixture of toxin and antitoxin, i.e., a slightly toxic mixture, should be used and should represent about 85–90% of an L+ dose of toxin to each unit of antitoxin. The mixture should be prepared with a diphtheria toxin of such strength that each dose of 1 cc. of the finished mixture will contain approximately 0.1 L+ dose of toxin almost neutralized with antitoxin. Three subcutaneous injections of 1 cc. each at intervals of about a week are required for immunization. The toxin-antitoxin mixture can be bought ready for injection. In an epidemic one could first give to those showing Schick reactions an immediate prophylactic injection of antitoxin to be followed three weeks later by 3 injections at seven-day intervals

of the toxin-antitoxin immunizing mixture. Immunity is acquired in about 2 months as shown by Schick test. The duration of the immunity in at least 90% of children is for more than six years and probably for the remainder of life. There seems to be no difference in this respect between those so immunized and those who develop antitoxin naturally.

It has been found that when diphtheria toxin is subjected to the action of formalin at 37°C., the toxic properties are destroyed while the immunizing properties are retained. This product, known as diphtheria toxoid (anatoxine) has been used in the immunization of children with good results.

Laboratory diagnosis.—In obtaining material from a throat, be sure that an antiseptic gargle has not been used just prior to taking the culture. The part of the swab which touched the membrane or suspicious spot should come in contact with the serum slant. This is best accomplished by revolving the swab. An immediate diagnosis is possible in probably 35% of cases by making a smear from a piece of membrane. In doing this, Neisser's stain or the toluidin blue stain are usually considered the most satisfactory. I prefer the Gram stain, however. The diphtheria bacilli found in such smears are not apt to be clubbed and stain more uniformly than those taken from young cultures.

If there is any doubt about the nature of an organism in a throat culture, always stain: 1. With Löffler's alkaline methylene blue for two minutes; 2. with Gram's method, being careful not to carry the decolorization too far, and 3. by Neisser's method. With well aged Löffler's stain you obtain a picture which, after a little experience, is characteristic; at times the polar bodies show as intense blue spots in the lighter blue bacillus. One is liable to mistake cocci lying side by side for diphtheria bacilli with segmental or banded staining. This difficulty is not apparent when Gram's staining is used. This gives us great information, as the diphtheria and the pseudo-diphtheria are the only small Gram-positive bacilli usually found in the throat. The cocci are also well brought out. Neisser's stain, when satisfactory, gives a picture which may be absolutely characteristic, bright blue dots lying at either end of light brownish-yellow rods. When first isolated from a throat, the diphtheria bacillus is apt to stain characteristically by Neisser. Later on, in subculture, there may be no staining of the polar bodies. Neisser originally recommended five seconds' application, with an intermediate washing, for each of his two solutions. Thirty seconds for each is probably preferable. Some authorities recommend five to thirty minutes. It is well to bear in mind that about 2% of persons in apparent health carry diphtheria bacilli of the granular or barred type in their throats and of these about one in five will prove virulent for the guinea pig.

Virulence test.—It is essential when a question exists as to the nature of a diphtheria-like organism to test it as to virulence. While there are exceptions, especially in freshly isolated colonies, yet as a rule a severe infection yields virulent organisms and *vice versa*. Pure cultures are best obtained by streaking material from the throat on glycerin agar or blood agar plates. From an isolated colony inoculate a tube of bouillon. From such a forty-eight or seventy-two-hour-old culture inoculate a guinea pig with 2 or 3 drops subcutaneously in the shaven abdomen. Escherich considers a fatal result with 1.5 cc. of such a bouillon culture a satisfactory test as to virulence. After death, which occurs in two or three days, the adrenals are enlarged and haemorrhagic.

Kolmer and Moshage recommend that a pure culture of the organism to be tested be grown on a slant of Löffler's blood serum, washed off with 10 cc. of sterile salt solution and 4 cc. of the suspension be injected subcutaneously in the median abdominal line of a 250–300 gram guinea pig.

The diagnosis is more sure if, in addition to the first animal, a second one, which has had antitoxin, is inoculated. The protected one should live.

Diphtheroid Bacilli. Pseudodiphtheria Bacillus. Hoffmann's Bacillus.—Under these terms various Gram-positive bacilli have been described as occurring in genito-urinary, nasal and skin diseases.

Their chief importance is in connection with their presence in the throats of healthy people. Probably approximately 10% of people harbor such organisms as against 1 to 2% with granule types. Some authorities believe it possible for these diphtheroids to be transformed into virulent diphtheria bacilli. This seems improbable. Such organisms are often found in urethral discharges, either alone, or with gonococci or other organisms.

A great deal of attention has been given to the etiological relationship between diphtheroids and Hodgkin's disease. Fox, in a critical study of this relationship, has obtained diphtheroids of varying morphology and cultural characteristics from the glands in Hodgkin's disease as well as from enlarged glands in chronic atrophic arthritis and other conditions. It would appear conservative to reject diphtheroids as causative agents not only in this disease but in leprosy as well.

Negri has applied the name *Corynebacterium granulatis maligni* to diphtheroids isolated from glands in Hodgkin's disease. The granular rods of Much, supposed to be connected with tubercle bacilli, may be diphtheroids. Mallory has connected diphtheroids with scarlet fever.

Diphtheroid characteristics.—1. These bacilli very rarely give the blue-dot staining at the two ends. Exceptionally they may give a dot at one end. Neisser attaches importance to the dots at both ends as showing diphtheria.

2. They tend to stain solidly or at most with only a single unstained segment. They are shorter, thicker, and do not curve so gracefully as the true diphtheria bacillus. They are stockier.

3. They produce very little acid in sugar media, not one-half that produced by true diphtheria. Goldberger found 29 out of 30 cultures of *B. diphtheriae* virulent and acid producers. Of 47 Hoffmann cultures 6 showed slight acid production while 41 produced alkali. All were nonvirulent.

4. They are nonpathogenic for guinea pigs.

5. Many of them grow quite luxuriantly and often show chromogenic power.

Xerosis Bacillus (*Corynebacterium xerosis*).—This organism is frequently found in normal conjunctival discharges. There is question as to its pathogenicity, and the finding of this organism should not exclude search for strictly pathogenic organisms, such as the *Gonococcus* or the Koch-Weeks bacillus.

Bacillus xerosis resembles the diphtheria bacillus in being Gram-positive and showing parallelism, but differs (1) in being nonvirulent for guinea pigs; (2) in requiring about two days for the appearance of colonies; (3) in not showing Neisser's granule staining, and (4) in producing very little acid in sugar media.

Fermentation reactions of the diphtheria and diphtheroid group, according to Knapp (modified.)

Species	Dextrose	Saccharose	Dextrin
B. diphtheriae	+	−	+
B. xerosis	+	+	−
B. hofmanni	−	−	−

(Note.—One per cent. sugar in Hiss serum-water media).

Bacillus mallei (*Pfeifferella mallei*) Löffler and Schütz, 1882.—This is the cause of a rather common disease of horses. When affecting the superficial lymphatic glands, it is termed "farcy;" when producing ulceration of nasal mucous membrane, the term "glanders" is used. In glanders, involvement of various organs, especially the lungs, tends to follow the primary lesions of the nasal mucosa and adjacent glands.

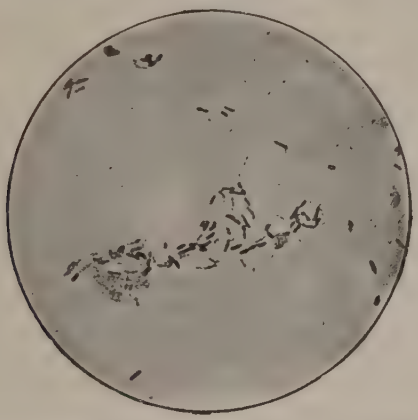


Fig. 36.—*Bacillus mallei* from an agar culture. ×1100. (*After Park and Williams.*)

In man there are 2 types of glanders—chronic and acute. In the chronic form an abrasion becomes infected from contact with glanders material and an intractable foul discharging ulceration results. There may be an involvement of nasal mucosa as well as skin. This may persist for months with lymphatic involvement or may become acute. The acute form may also develop from the start and the cases are usually diagnosed as pyaemia. There is great prostration with marked pains of the

extremities. Pustular lesions, resembling those of smallpox, may be present. Death almost invariably results in acute glanders.

Morphology and cultural characteristics.—The bacillus is a narrow, slightly curved rod, about $3 \times 0.3\mu$. It is nonmotile and Gram-negative. It at times presents a beaded appearance and tends to show irregularity of staining. In subculture on agar or blood serum the growth is somewhat like typhoid but more translucent. In original cultures from pus or tissues the colonies may not show themselves for forty-eight hours. The optimum temperature for culturing is 37.5°C.

As the organism does not tend to invade the blood stream, blood cultures are apt to be negative. The glanders bacillus grows best on an acid glycerin agar (+2). Bouillon cultures show a slimy sediment and surface pellicle. Milk is slowly coagulated and the litmus of litmus milk turned pink.

The characteristic culture is that on potato. Grown at 37°C., we have a light brown or yellowish honey-like or mucilaginous growth, which by the end of a week spreads out and takes a cuprous oxide-like reddish tint with greenish borders. The potato assumes a dirty brown color. Cultivation on potato and the inoculation of a guinea pig are the chief diagnostic measures. If the material is injected intraperitoneally into a male guinea pig, marked swelling of the testicles is noted from within forty-eight hours, at the earliest, to ten days. Cultures should be made from the testicle as other organisms than glanders may cause the swelling.

Only the *B. pyocyaneus* and cholera vibrios give a similar coloration of potato. These organisms, however, are easily differentiated. Glanders is the most dangerous of laboratory cultures and should be handled with extreme care.

Staining.—The best stains are carbol thionin and carbol fuchsin. In sections stained with carbol thionin the bacilli are apt to be decolorized by the subsequent passage of the section through alcohol and xylol. This may be avoided by blotting carefully after the thionin, then clearing with xylol or some oil and mounting. Nicolle's tannin method is a good one.

Mallein is prepared by sterilizing cultures, that have grown in glycerin bouillon for about five months, by means of heat (100°C.). The dead culture is then filtered through a Berkefeld filter and the filtrate evaporated to one-third of its original volume and subsequently made up to its original volume with a 10% glycerin solution containing 1% carbolic acid. It is chiefly used as a means of diagnosing the disease in horses. The reaction consists in rise of temperature and local oedema. The dose is about 1 cc.

Agglutination and complement fixation tests are also used for diagnosing glanders.

Bacterium whitmori.—This organism is the cause of a rare, glanders-like disease, described by Col. Whitmore of the British Army, found at autopsies of beggars in Rangoon. Stanton and Fletcher suggested the name melioidosis, in order to describe its close relationship to glanders. *B. whitmori* closely resembles *B. mallei*, being a small

Gram-negative bacillus about the same size and shape, and occurring in very large numbers in the acute lesions of the disease.

On culture it closely resembles the glanders organism, but it is more actively motile and liquifies gelatin more rapidly. It grows luxuriantly and forms a dense wrinkled culture on glycerin agar. In guinea pigs the infection is more rapidly fatal than is glanders; producing in the male guinea pig an acute orchitis—the so-called Strauss reaction. The organism is excreted in the urine and faeces of infected laboratory animals. Several cases of natural infection have been found in rats, and one case in a domestic cat, has been observed by Stanton.

CHAPTER VIII

STUDY AND IDENTIFICATION OF BACTERIA—GRAM-NEGATIVE BACILLI. KEY AND NOTES

KEY to the recognition of nonspore-bearing, nonchromogenic, non-Gram-staining, nonbranching bacilli.

I. Do Not Grow on Ordinary Media.—Minute rod-shaped cells, sometimes thread forming and pleomorphic. Grow best (or only) in the presence of haemoglobin and in general requiring blood serum, ascitic fluid, or certain growth accessory substances. Nonmotile.

A. Aerobic.

(a) Affecting the respiratory tract.

(1) Hemophilus influenzae (Pfeiffer influenza bacillus).

(2) Hemophilus hemolyticus (haemolysis in blood broth. Found in upper respiratory tract. Nonpathogenic).

(3) Hemophilus pertussis (Bordet-Gengou bacillus of whooping cough).

(b) Affecting the conjunctiva.

(4) Hemophilus conjunctivitidis (Koch-Weeks bacillus of acute infectious conjunctivitis).

(5) Hemophilus lacunatus (Morax-Axenfeld bacillus of subacute infectious conjunctivitis or "angular conjunctivitis").

(c) Affecting the genital region.

(6) Hemophilus ducreyii (Ducrey's bacillus of chancroid).

(7) Hemophilus canis (preputial secretions of dogs).

B. Anerobic.

(1) Dialister pneumosintes (*Bact. pneumosintes* of Olitsky and Gates). Occurs in the nasal secretions of influenza patients in the early hours of the disease. Very short rods with pointed ends occurring singly and in pairs. Strict anaerobe. Filter passer.

II. Grow Scantily or Require Enriching Substances in Media.

(1) Pasteurella tularensis (*Bact. tularense*) growth occurs on media containing egg yolk, cystine or blood agar. Fresh sterile rabbit spleen favors the growth.

(2) Alcaligines melitensis (*Bact. melitense*, Bruce) causes Malta fever. Distributed usually through goat's milk.

(3) Alcaligines abortus (*Bact. abortum*, Bang) causes contagious abortion in cattle; also reported in human infections. Recent studies on the serologic relations of A. abortus and A. melitensis show that these two organisms are closely related, if they are not identical.

III. Grow Well on Ordinary Media.

A. Cultures in litmus milk. PINK.

(Commonly occurring in intestinal canal of man and animals. Produce acetyl-methyl-carbinol.—Voges-Proskauer reaction).

(a) Nonmotile.

(1) Aerobacter aerogenes (*B. lactis aerogenes*) produces acid and gas in adonitol, lactose, dextrose and sucrose. No action on dulcitol. Indol not formed.

(b) Motile.

(2) Aerobacter cloacae (*B. cloacae*) ferments lactose and sucrose. No action on adonitol or dulcitol. Indol formation indefinite. Slow liquefaction of gelatin. Commonly occurring in intestinal canal of man and animals. Generally attacks carbohydrates, forming acid and often gas composed of CO_2 and H_2. Acetyl-methyl-carbinol formed from dextrose.

(3) Escherichia (*B. coli* group). Representative type species are:

(a) Escherichia coli (*B. coli communis*) acid and gas are formed from glucose, lactose and dulcitol; not from sucrose. Indol produced. A "commensal" species.

(b) Escherichia communior (*B. coli communior*) similar to E. coli except acid and gas is formed from sucrose.

B. Cultures in litmus milk. LILAC.

(a) Nonmotile.

(1) No gas generated in dextrose or lactose bouillon.

(aa) Haemorrhagic septicaemia group.

(1) Pasteurella pestis (*B. pestis*). Causative organism of plague in man, rats and ground squirrels. Oval bacilli with tendency to bipolar staining. Agar colonies grayish white, translucent, iridescent, undulate. Several species of the genus Pasteurella are pathogenic for domestic and wild animals (fowl cholera, swine plague, etc.).

(bb) Dysentery group. Attack a number of carbohydrates with formation of acid, but no gas.

(1) Eberthella dysenteriae (*B. dysenteriae*, Shiga). The cause of the bacillary dysentery in man.

(2) Eberthella paradysenteriae (Hiss, Flexner, Strong types). The cause of dysentery in man especially of the so-called "Summer diarrhoea," of infants.

Note: Dysentery organisms may be differentiated by serological tests in the form of the agglutination reactions and the absorption of agglutinins and on the basis of the following cultural reactions:

	Dextrose	Mannitol	Maltose	Sucrose	Indol
Dysenteriae (Shiga)....	+	−	−	−	−
Hiss type.....	+	+	−	−	+
Flexner type.....	+	+	+	−	+
Strong type.....	+	+	−	+	+

(2) Gas generated in dextrose and sometimes lactose bouillon.

(aa) Friedlander group. Ferment a number of carbohydrates with formation of acid and gas. No liquefaction of gelatin. Encapsulated. Found principally in respiratory tract of man.

(1) Klebsiella pneumoniae (*Pneumobacillus*, Friedlander). Agar colonies, white, very viscid, opaque, shiny, convex. Associated with pneumonia and other inflammations of the respiratory tract.

(2) Klebsiella granulomatis (*Calymmatobacterium granulomatis*). Closely resembles Friedlander's organism. Found in lesions of granuloma inguinale.

(3) Klebsiella rhinoscleromatis. Given as cause of rhinoscleroma.

(4) Klebsiella ozaenae (*B. ozaenae*). Given as cause of ozena. Infectious for house and field mice.

(b) Motile.

(1) Do not liquefy gelatin.

(aa) Typhoid group. Attack a number of carbohydrates with formation of acid but no gas.

(1) Eberthella typhi (*B. typhosus*, Eberth.). The cause of typhoid fever. Pathogenic for laboratory animals by subcutaneous or intravenous injection. Indol not produced. Agglutination with immune serum. Several species similar to Eberthella typhi described but are nonpathogenic for man (E. enterica, E. oxyphila, etc.).

(bb) Salmonella Group. Attack numerous carbohydrates with formation of acid and gas. Acetyl-methyl-carbinol not formed. Occur in intestinal canal of man and animals. Certain types pathogenic. Cause salmonellosis (food poisoning) and paratyphoid fevers in man.

(1) Salmonella schottmülleri (*B. paratyphosus B*). No acid or gas in lactose bouillon. The cause of paratyphoid fever. Occurs in food poisoning where meat from infected animals is used.

(2) Salmonella aertrycke (*B. aertrycke*). Encountered in continued fevers of paratyphoid type. Meat poisoning.

(3) Salmonella typhimurium associated with a fatal epidemic in mice.

(4) Salmonella enteritidis (*B. enteritidis*, Gaertner). First isolated from intestines in epidemic of meat poisoning. Occurs in domestic and wild animals.

(5) Salmonella paratyphi (*B. paratyphosus A.*).

(6) Salmonella morgani (*Bact. morgani*). Found in intestinal canal of dysentery patients.

(2) Liquefy gelatin.

(aa) Proteus group. Highly pleomorphic rods, filamentous and curved. Produce characteristic amoeboid colonies on moist

media and decompose proteins. Ferment dextrose and sucrose but not lactose. Do not produce acetyl-methyl-carbinol.
(1) Proteus vulgaris—Liquefies gelatin rapidly.
(2) Proteus mirabilis—Liquefaction of gelatin.
(3) Proteus asiaticus does not liquefy.

Note: Proteus X_2 and X_{19} used in Weil-Felix reaction belong to the above species. Isolated from urine of patients suffering from typhus fever.

Gram-negative Bacilli Requiring Special Media

Bacillus influenzae (*Hemophilus influenzae*) Pfeiffer, 1892.—This organism is the type of the so-called haemophilic bacteria—organisms whose growth is restricted to media containing haemoglobin. The blood agar plate is very convenient for the isolation of *B. influenzae* as it enables us readily to differentiate haemolytic and green-producing colonies.

The influenza bacilli are most likely to be isolated from the sputum of bronchopneumonia due to this organism. It has also frequently been found in the nasal secretions of influenza patients. Exceptionally, it is present in the blood, and has been isolated in cases of meningitis from cerebrospinal fluid. It also occurs at times in anginas, but then usually associated with other organisms. It is a very small bacillus which in sputum tends to show itself in aggregations, especially centering about *M. tetragenus*. It stains rather faintly when compared with cocci, so that a smear of sputum stained with formol fuchsin shows a deep violet staining for the *M. tetragenus* or other cocci, and scattered around in a clump-like aggregation we see these minute, rather faintly stained rods. They also tend to stain more deeply at either end, so that they sometimes appear as diplococci. Gram's method, counterstaining with formol fuchsin, is excellent for their demonstration. Although usually reported as small rods $.5 \times .25\mu$, they frequently, when first isolated, show involution types more or less suggesting the bizarre forms of plague bacilli on salt agar. Filamentous forms also have been noted. Some regard such forms as indicating virulence. The Pfeiffer bacillus is nonmotile and but rarely forms chains. Organisms resembling the Pfeiffer bacillus have been isolated from cases of whooping-cough, and have been found also in the fauces of well persons.

Culture media.—Apparently the best medium for isolating the Pfeiffer bacillus is Avery's oleate haemoglobin agar. More luxuriant growth is obtained by using blood agar plates made by adding the blood to the agar at 90° to 100°C. (cooked blood or chocolate agar) rather than as ordinarily prepared, but such plates are opaque and less satisfactory for isolation. The material should be smeared or streaked over the surface of blood agar.

The colonies appear as very minute dewdrop-like points which require a lens to be distinctly seen at the end of 24 hours, but after 48 hours they show as small, transparent, spherical spots, the size of a small pin's head. To test the identity of such colonies we should transfer a single colony to plain agar and blood serum, on

which Pfeiffer's bacillus will not grow, trying not to carry over with it any blood since the least trace would permit growth. The influenza bacillus seems to grow best in symbiosis with some other organism, especially with *S. aureus*. It has, as a rule, very slight virulence for experimental animals although rarely a strain may be encountered which is virulent for guinea pigs.

Immunity.—An attack of influenza does not seem to give immunity, although a transient lack of susceptibility following an attack must be the explanation of the usually observed succession of outbreaks over two or three years. The first outbreak usually gives mild cases while the succeeding ones are attended with greater mortality. Secondary infections with various pneumonia-causing organisms seem responsible for most of the deaths. Not infrequently influenza involves the lungs and the organism may cause a septicaemia, otitis, meningitis or even conjunctivitis and pyelitis.

Consumptives are liable to become carriers of the Pfeiffer bacillus, the organism being present in the sputum of such patients for long periods of time.

Etiology of influenza.—As the result of the investigations of the etiology of influenza during the 1918 pandemic it may be stated that we must consider this problem as unsolved. In many epidemics of influenza the Pfeiffer bacillus has not been isolated, or success has obtained in only a small proportion of cases. Rosenau and others tried in every way to infect persons with the influenza bacillus and with various discharges and filtrates from patients with influenza without success. The existence of strains of influenza bacilli has been demonstrated but there has been nothing shown as to agreement of strain in contact groups. Various workers have shown that the Pfeiffer bacillus is present in the throats of normal individuals, as well as in those of patients with other diseases, in about the same proportion before and since the pandemic as during that period. For example, Sellards isolated the Pfeiffer bacillus from the sputum and conjunctival secretions from 25 of 31 cases of measles. The findings in lung punctures during life and culturing lungs at autopsy following influenza pneumonia have not shown any constancy in the isolation of the Pfeiffer bacillus. Careful examination of sputum, as well as throat and nasal discharges from typical cases of influenza, has failed to demonstrate any constancy of bacteriological finding. The reports as to the presence of agglutinating substances in the blood of influenza patients are contradictory.

Etiological factors in clinical conditions more or less resembling influenza may be the *Streptococcus*, *Pneumococcus*, or *M. catarrhalis*. A coccus, known as the Mather's coccus, which resembled a type IV pneumococcus morphologically, but differed in not being bile-soluble, was isolated from sputum and lungs of influenza cases during the recent pandemic. In 78 autopsies on post-influenzal pneumonia cases in the A.E.F., Holm and Davison obtained pure cultures from lungs of *B. influenzae* 7 times, type IV *pneumococci* 11 times, *S. haemolyticus* 9 times, and *Meningococcus* 7 times. In 23 cases *B. influenzae* was found with other organisms. Apparently vaccination with organisms other than the Pfeiffer bacillus has given about the same doubtful results in prophylaxis as where mixed strains of the Pfeiffer bacillus have been employed.

In view of the reasonable doubt generally entertained regarding the existence of a causal relationship between the *B. influenzae* and the disease influenza, it would appear well to change the name of this organism to Pfeiffer bacillus.

However Blake and Cecil reported successful results by inoculating monkeys intranasally with a strain of *B. influenzae*, which had been raised in virulence by a passage through animals. Such a strain (.01 cc. of a blood broth culture) would kill a white mouse in 48 hours. The disease in the monkey seemed identical with influenza in man. They also produced mild systemic manifestations, but without fever, in human volunteers.

Influenza has become so intimately associated in our minds with manifestations due to secondary invaders, chiefly respiratory types, that we rarely recognize the uncomplicated disease except in times of epidemic. The abrupt onset in a period of normal well-being, the rapid rise of fever to 100°F. or higher, the headache, pains in back and muscles of the calves of the legs and weariness of the eyeballs make a picture very similar to that of the dengue-like fevers. Further, we may have as with dengue a distinct tendency to leukopenia, marked prostration, physical weakness in convalescence and even an eruption. Such a picture of influenza is not generally carried in mind because we demand such manifestations as cough and bronchial catarrh in a typical case, not recognizing that these respiratory symptoms may not improbably arise from secondary infection with familiar inciters of respiratory trouble—among these the Pfeiffer bacillus.

In regard to the views frequently expressed concerning a filterable virus as the cause of influenza, it should be noted that the views of Nicolle and Lebailly as to the filterability of the virus of influenza (as well as those of Gibson and others) do not seem to be in accord with the investigations of American workers, but Olitsky and Gates report that they have infected rabbits with intratracheally inoculated filtrates from the secretions of early cases of influenza. It is possible that in the filtrates obtained by the French investigators there may have been present the filter-passing organism of Olitsky and Gates to which the name *Bacterium pneumosintes* has been given. This organism, obtained from the nasopharyngeal secretions in the early hours of uncomplicated epidemic influenza, has been described as a minute bacilloid body, 0.15 to 0.30μ in length, larger forms being noted in certain media. It grows only in media enriched with fresh animal tissue or defibrinated rabbit blood, and under anaerobic environment. Original cultures are best made from filtered nasopharyngeal secretions, since the bacillus passes Berkefeld V and N filters. In rabbits intratracheally inoculated, a febrile disease with leukopenia and pulmonary oedema and emphysema was produced, and the way seemed to be opened for the invasion of streptococci, pneumococci and the Pfeiffer bacillus. Specific agglutinins, of low titre, however, are noted in the blood of experimentally infected animals.

Koch-Weeks Bacillus (*Hemophilus conjunctivitidis*) Koch, 1883.—This produces a severe conjunctivitis. It is very common in Egypt and is also a frequent cause of conjunctivitis in the Philippines and in temperate climates. The conjunctivae of animals, other than man, resist infection with this bacillus. Flies are an important etiological factor in Egypt. The incubation period of the disease is short, 12 to 36 hours.

Smears made from conjunctival secretion in cases of this infection show large numbers of small Gram-negative bacilli, especially contained within pus cells, but also lying free. They are nonmotile, somewhat longer than the Pfeiffer bacilli ($.25 \times 1.5\mu$), and more difficult to cultivate, but the same general methods hold. The vitality of this organism is very slight so that almost immediate transference of material is necessary. The best medium is a mixture of glycerin agar and hydrocele or ascites fluid. At first we rarely obtain pure cultures. The colonies are dew-drop-like and first show themselves in about 36 hours in incubator cultures.

It would seem that blood agar is a better medium than a serum one. Many haemoglobinophilic bacteria will grow with only 1 to 500 haemoglobin, so that growth on serum might be explained by slight blood admixture.

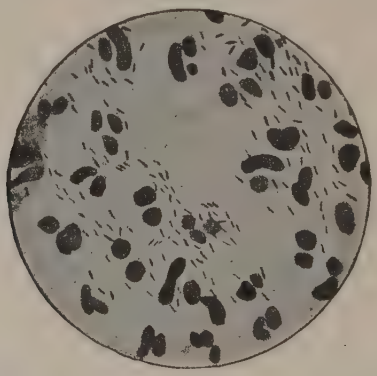


FIG. 37.—The Koch-Weeks Bacillus. (*Hansell and Sweet.*)

Nature of inclusion bodies of trachoma.—Some have thought that repeated infection with the Koch-Weeks bacillus was the cause of trachoma. Others have regarded other haemoglobinophilic bacteria as causative. According to the views of Park and Williams the inclusion bodies of Prowazek, supposed to be characteristic of trachoma, are simply clumps of extremely small, coccoid haemoglobinophilic bacteria. Besides these organisms the *Gonococcus* in smears from gonorrhoeal ophthalmia is stated to show involution forms having a resemblance to trachoma bodies.

Noguchi thinks that while there is a morphological similarity between degenerated haemoglobinophilic bacteria and cell inclusions, yet in the latter, the elementary bodies are much smaller than the bacterial granules, and the initial bodies less definite in contour. He was able to infect the conjunctivae of monkeys with inclusion-bodies material, but not with haemoglobinophilic bacilli. See page 660.

Diplobacillus of Morax (*Hemophilus lacunatus*).—This organism causes mild blepharoconjunctivitis chiefly at the internal angle of the eye. The bacilli are about 1 or 2μ long by about 1μ in width and tend to occur in pairs or short chains. They are nonmotile and may show

involution forms, especially after 2 or 3 days on blood serum. Some claim that they are Gram-positive.

Culturally, the formation of little pits of liquefaction on Löffler's serum within twenty-four hours which later become confluent may be regarded as fairly characteristic. They do not grow on nutrient agar.

While usually causing a more or less chronic conjunctivitis they may at times produce a keratitis.

NOTE.—A Gram-negative bacillus which is less than 1 micron long, growing singly, or in pairs, and known as the bacillus of Zur Nedden has been stated to produce corneal ulcers. It grows readily on agar or other ordinary culture media. It coagulates milk.

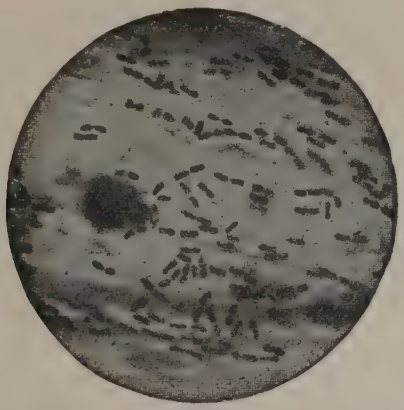


FIG. 38.—The diplobacillus of Morax in the exudate of conjunctivitis. (*From McFarland after Rymowitsch and Matschinsky.*)

Bacillus of Chancroid (*Hemophilus ducreyii*) Ducrey, 1889.—This is often called the streptobacillus of Ducrey on account of its occurring in chains, but when ulceration has set in, chain-formation is not observed.

The organism is Gram-negative, shows bipolar staining and is about .5 × 1.5μ. Aspirating the gland juice with a hypodermic syringe from the buboes which are apt to follow chancroid offers the best means of obtaining pure cultures. This should be done before the bubo suppurates. Ducrey's bacillus being exceedingly delicate, the syringe should be warmed to body temperature and the transfer made to media at the same temperature and at once put in the incubator. Cultures kept at room temperature quickly die, but in the incubator remain alive for a week or more. Cultures and smears can also be made from the lesion, preferably before it ulcerates. Teague and Diebert got 140 positive cultures in 274 cases by the following method: Blood from a rabbit's heart was deposited in small tubes in 1 cc. quantities. After the blood had clotted, the tube was heated at 55°C. for 5 minutes to

destroy natural bactericidal substances. The serum about the clot was inoculated from the chancroidal sore or bubo and after 24 hours' incubation smears were made and stained for small, Gram-negative bacilli in chains. Small glistening gray colonies, which slide over the surface when touched with the platinum loop, may be grown by transfer to blood agar plates.

Bacillus of Bordet-Gengou (*Hemophilus pertussis*).—This bacillus was reported as the cause of whooping-cough by Bordet and Gengou in 1906. (Czaplewski and Reyher had previously reported oval bipolar-staining organisms, as the cause of pertussis, and other authors influenza-like organisms.)

The bacillus is small (.3 × 1.2μ), oval, Gram-negative, shows bipolar staining, somewhat resembles *B. influenzae* (Pfeiffer) and grows only on uncoagulated serum media, as blood or ascites agar. The original cultures are very scanty so that the colonies are difficult to recognize. In subcultures the growth is more flourishing. The organism is found only in the white, thick, leukocyte-abounding sputum of the beginning of the disease. Hence pertussis is probably contagious only at the onset.

Complement binding and agglutination reactions have been obtained. For diagnosis stain the sputum. Remember that pertussis gives a mononuclear leukocytosis of 15,000 to 50,000.

Special culture medium.—For isolation from sputum the following medium is required. Autoclave 500 grams potato with 1000 cc. of 4% glycerin solution. Pour off excess of fluid. Emulsify potato in 1500 cc. normal salt solution and add powdered agar to 3 or 4%. For use mix with an equal quantity of defibrinated blood.

It is not entirely settled that *B. pertussis* is the cause of whooping-cough. It must be remembered that in culturing sputum from a case we may obtain a Gram-negative bacillus, which, however, shows a profuse growth even on ordinary agar. Monkeys and young dogs have been injected with cultures of the pertussis organism; the dogs however die apparently of canine distemper. It has been suggested that dogs may spread the disease but this seems improbable. A lasting immunity is conferred. The value of vaccines of this organism in prophylaxis is questionable and their use in treatment apparently unsuccessful.

Bacterium tularense (*Pasteurella tularensis*) McCoy and Chapin, 1911.—This organism causes a plague-like disease in various rodents and is transmissible to man by (1) the bite of a horsefly, *Chrysops discalis*, (2) by the tick *Dermacentor andersoni* which have fed on jack rabbits or ground squirrels infected with tularaemia, and (3) by contamination of hands or conjunctival sac with portions of internal organs or body fluids of infected rabbits. In man we have an irregular fever lasting 2 or 3 weeks, and, when there has been a fly-bite, an ulcer at this point and enlargement of tributary glands. Cases have been

reported from 22 states and the District of Columbia. Ohara has reported this disease from Japan and his work was confirmed by Francis.

Bacterium tularense is a small, nonmotile Gram-negative organism from 0.3 to 0.7μ long, and gives the appearance of being surrounded by capsular material in stained smears from tissues; it also has a coccus form. See Fig. 39 illustrating the two forms. The organism stains best in tissue preparations with Giemsa solution but in smears from cultures it takes aniline gentian violet very readily.

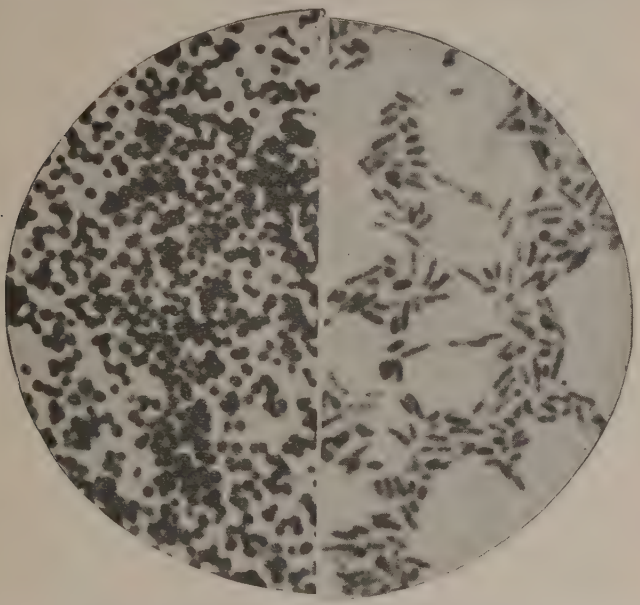


FIG. 39.—*Bacterium tularense*. Note change from coccus-form to rod-form in a single transfer on culture medium (Photomicrograph by Major G. R. Callender, M.C., U. S. A., by courtesy of Surgeon E. Francis, U.S.P.H.S.).

It will not grow on plain agar or in bouillon but at first was cultivated only upon coagulated hen's egg yolk. Francis has grown the organism on blood agar, glucose blood agar and serum glucose agar, but growth on these media *per se* is scanty. The growth, however, is greatly enhanced if the medium is supplemented by a piece of fresh sterile tissue; this tissue may be supplied by the piece of spleen of the infected rabbit or guinea pig with which the medium is inoculated, or the medium may be prepared for subcultivation by rubbing over the surface of the plain medium a piece of sterile spleen (about 3 mm. in diameter) from a healthy rabbit and allowing the piece to remain on the surface of the medium.

Serum glucose cystine agar (Francis).—Serum glucose cystine agar has taken precedence as a satisfactory medium both for first isolations and for subcultures.

The cystine medium is inoculated with the animal's heart blood or small piece of the liver or spleen of the infected animal is rubbed on the surface of the medium and allowed to remain. Growth appears about the third day, which, in subcultures, grows luxuriantly on the serum glucose cystine medium without the addition of fresh tissue.

Cutaneous infection of a guinea pig is possible as for plague. At autopsy an infected guinea pig shows haemorrhagic oedema at the site of inoculation, with caseation of lymph glands and small necrotic foci in spleen and liver. Smears from the spleen usually show the bacilli.

Laboratory infections.—There have been 18 cases of laboratory infection of man and this is a particularly dangerous organism to work with, although death from the disease in man rarely follows infection.

Market infections.—Up to the present time there have been 47 cases of the ulcero-glandular type of tularemia reported among market men from dressing rabbits. The organism has been isolated from livers of rabbits offered for sale at market stands.

Diagnosis.—Inoculate material from the patient's ulcer, glands or blood into guinea pigs, rabbits or mice from which the organism may be isolated on special media. The organism is rarely present in the blood of human cases. Complement fixation and agglutination tests are the best methods of laboratory diagnosis in the absence of animal inoculations. In the Hygienic Laboratory an antigen is prepared by washing off the 48-hour growth on serum glucose cystine agar with a small amount of saline. The suspension is heated for 30 minutes at 56°C. and then preserved by the addition of 0.3% trikresol. Such an antigen is used for both types of test.

Bacterium melitense (*Alcaligines melitensis*) Bruce, 1887.—Some use the generic name *Brucella*. This is the organism of undulant fever, so called on account of successive waves of pyrexia extending over several months. (Synonyms: Malta or Mediterranean fever.) The disease has a very slight mortality (2%), and the lesions are chiefly of the spleen, which is large and diffluent. The organisms can best be isolated from the spleen or blood.

Morphologically, the *Bact. melitensis* is a very short rod when studied in smears made from the spleen. In cultures it is rather coccobacillary in form and may appear as a distinct bacillus, which may form short chains in broth cultures.

Its characteristics are its very small size and the dew-drop minute colonies on agar, which at incubator temperature show themselves only about the third to the sixth day. It is nonmotile and Gram-negative. In bouillon there is a slight turbidity. Gelatin growth is very slow and there is no liquefaction. Litmus milk becomes more blue after a week, so that there is an alkaline action. Indol is not produced. There is no fermentation of glucose, maltose, mannite, lactose or saccharose. The optimum reaction of media is +0.8. It grows best at 38°C.

Recent studies on the serologic relations of *A. abortus* and *A. melitensis* show that these two organisms are closely related, if they are not identical.

Evans has shown that *Brucella melitensis* variety *abortus* may infect man.

The disease of man contracted from cattle or hogs is indistinguishable clinically from that prevalent in Mediterranean countries and known as undulant, or Malta fever. (Abortus type of undulant fever.)

The bovine and caprine varieties of the organism causing undulant fever are so closely related that they cannot be distinguished by ordinary laboratory tests. By the agglutinin absorption test, however, a slight but distinct difference between the two varieties may be detected. They are more closely related than the serologic types of meningococcus.

Many laboratory infections have been recorded.

The organism occurs in the peripheral circulation, it having been cultivated very successfully by Eyre from blood taken at the height of the fever, and in the afternoon. Formerly it was customary to obtain it by splenic puncture.

Infection is acquired chiefly from milk of infected goats. The organisms are excreted in the urine of patients, and a diagnostic method is to make plates from the urine. Such urine applied to abraded surfaces may cause infection.

The serum of patients shows agglutinating power as early as the fifth day of the disease, and this power may persist for years after recovery. Nicollę has advised using serum heated to 56°C. for thirty minutes for the agglutination test, nonspecific agglutinins being thereby destroyed. Carriers are not considered of much importance in the epidemiology of undulant fever.

A high mononuclear increase may be found in this disease.

Horses, cows and asses, as well as goats, are susceptible. It is very difficult to infect rabbits, mice and guinea pigs. Monkeys have been chiefly utilized in experimental work.

What may be deemed proof positive of goats' milk transmission is the practical disappearance of the disease among the naval and military forces of Malta, as the result of boiling the milk while still continuing among native civilians not boiling their milk. Bassett-Smith has noted that in 1905 there were 798 cases among civilians and 245 naval cases. In 1907 there were 457 cases among civilians and only twelve cases in the naval forces.

There are however occasional cases which Shaw has considered as due to carriers. As the organisms are excreted in faeces as well as in urine, and as the course of the disease is so protracted, as well as the convalescence, it would seem that the carrier factor should be of more importance than facts indicate.

Mohler has noted in Texas, where the disease has existed for twenty-five years, that the Mexican goatherds boiled their milk and hence were rarely infected.

The souring of milk does not destroy the germs of the disease; hence transmission may be brought about by butter and cheese.

Undulant fever was stamped out of Port Said by destroying all infected goats.

Infection may occur, (1) by the stomach atrium (usual), (2) by contaminated dust reaching lungs, and (3) by subcutaneous infection.

Gram-negative Bacilli Growing on Ordinary Media

Bacillus pneumoniae (*Klebsiella pneumoniae*) Friedländer, 1882.—This organism is responsible for about 5% of the cases of pneumonia. It is usually termed the *Pneumobacillus* to distinguish it from the *Pneumococcus;* again it is called *Bacillus mucosus capsulatus*, and at other times Friedländer's bacillus. The name of Fraenkel attaches to the *Pneumococcus*. Morphologically, the pneumobacillus is a short, thick rod ($1 \times 2.5\mu$) and in pathological material, as sputum, shows a wide capsule. It is nonmotile and Gram-negative. The colonies on agar are of a pearly whiteness and are markedly viscid. On potato it shows a thick viscid growth containing gas bubbles. The distinctive cultural characteristic is the "nail" appearance in a gelatin stab. The growth at the surface is heaped up like a round-headed nail, the line of puncture resembling the shaft of the nail.

It does not liquefy gelatin. It does not produce indol, and does not produce gas in lactose bouillon—points of difference from the colon bacillus, with which it may be confused in cultures, as it does not then possess a capsule. If in doubt, inject a mouse at the root of the tail. Death from septicaemia occurs in two days. The peritoneum is sticky and numerous capsulated bacilli are present in the blood and organs. The organisms which have been isolated from rhinoscleroma and ozaena are practically identical with the *B. pneumoniae*. In sections of the nodules of the nasal mucosa of rhinoscleroma are seen large cells with crescentic nuclei (Miculicz cells) which may be packed with these bacilli. Flu has reported capsulated organisms of the Friedländer group in cells of the granulomatous tissues of granuloma venereum. This group of organisms is generally referred to as the Friedländer group. Similar organisms have been isolated from the discharges of sinus infections, middle-ear disease and in anginas. Cases have been reported where the *B. pneumoniae* was the cause of septicaemia in man. The organism has been reported from cases of both lobar and lobular pneumonia, but such pneumonias are rare and, when occurring, tend to be fatal.

There are at least two types of Friedländer organisms, one represented by *B. lactis aerogenes* which produces gas in lactose as well as glucose media, while the organisms like *B. pneumoniae*, *B. rhinoscleromatis* and *B. oezaenae* produce gas only in glucose. A member of the group, the Perez bacillus, is stated to form indol.

Bacillus pestis (*Pasteurella pestis*) Kitasato, Yersin, 1894.—This, the organism of plague, was first isolated by Yersin from a plague bubo, in 1894, at Hong Kong. It is true that Kitasato reported a bacillus which he had isolated from the blood of a plague patient on July 7, 1894 (Yersin's report was made July 30, 1894), but his bacillus was motile, Gram-positive, coagulated milk and gave a turbidity in bouillon —characteristics which were just the opposite of those of the organism

reported by Yersin. It is primarily a disease of rodents, usually rats, but in some localities ground squirrels are the source of human infections. It is the member of the group of haemorrhagic septicaemias (Pasteurelloses) from which man suffers.

Other Pasteurelloses are chicken cholera, swine plague, mouse septicaemia and rabbit septicaemia. This is a widely distributed group and may include saprophytic organisms as well as those noted for their virulence.

B. cholerae gallinarum and *B. suisepticus* are approximately similar in size and cultural requirements to *B. pestis*. The appearance of an oval bacillus with bipolar staining in smears from tissues is very characteristic for both of them. Another name for swine plague (*B. suisepticus*) is infectious pneumonia of swine. The organism is chiefly found in the lungs.

Types of plague.—The two chief types are bubonic plague and pneumonic plague. Bubonic plague is characterized by the presence of exquisitely tender glandular enlargements of the inguinal (most common), axillary or cervical gland groups.

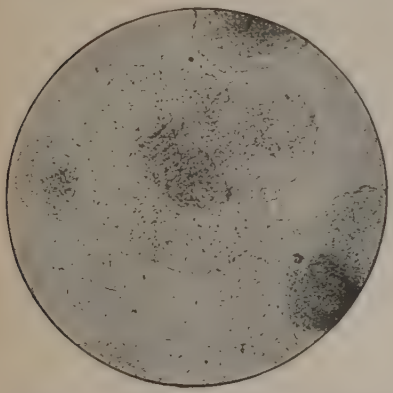


FIG. 40.—Colonies of plague bacilli 48 hours old. (*Kolle and Wassermann.*)

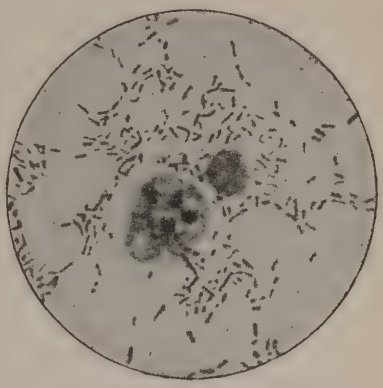


FIG. 41.—Pest bacilli from spleen of a rat. (*Kolle and Wassermann.*)

Pneumonic plague gives rise to a most prostrating bronchopneumonia, which invariably kills within 4 or 5 days. A plague bacteriaemia sets in with both the above types.

When the symptoms are slight, consisting mainly of buboes, the term pestis minor is sometimes used, the typical disease being termed pestis major.

We speak also of cellulo-cutaneous plague, when the bacilli are localized in the skin and subcutaneous tissue, and septicaemic plague when chiefly present in the blood.

Morphology and cultural characteristics.—In smears of material from buboes, from sputum, from blood or spleen smears from experimentally infected animals, or in blood smears, we obtain a coccobacillus ($1.5 \times .5\mu$), of typical morphology and with very characteristic bipolar staining, there being an intermediate unstained area.

Very characteristic also is the appearance in these smears of degenerate types which stain feebly and show coccoid and inflated oval forms. The presence of these involution forms associated with typical bacilli is almost diagnostic for one with experience. Inoculating tubes of plain agar with this same material, we obtain organisms which are typically small, fairly slender rods, which do not stain characteristically at each end and are not oval. Upon inoculating a salt agar slant with the culture from plain agar we obtain in the smears from the subculture remarkable involution forms—coccoid, root-shaped and sausage-shaped forms, ranging from 3 to 12 microns in length, more resembling cultures of moulds than bacteria. Another point is that on the inoculated plain agar we are in doubt at the end of 24 hours whether the dewdrop-like colonies are really bacterial colonies or only condensation particles. By the second day, however, these colonies have an opaque grayish appearance, so that now, instead of questioning the presence of a culture, we consider the possibility of contamination. Litmus milk is rendered slightly acid but not suffi-

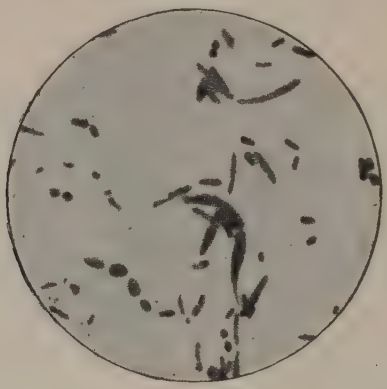


FIG. 42.—Pest bacillus involution forms produced by growing on 3% salt agar. (*Kolle and Wassermann.*)

ciently to change the lilac color. Glucose broth is made slightly acid but there is no effect on lactose. Ordinary bouillon cultures show a rather powdery deposit at the bottom, and a hanging-drop preparation from such a culture shows chains of plague organisms resembling streptococci.

Blood cultures in septicaemic plague may show from 5 to 500,000 bacilli per cc., but smears from the blood in such cases are positive in only about 17%.

The plague bacillus grows well at room temperature—its optimum temperature being 30°C. instead of 37°C., as is usual with pathogens. Next to the salt agar culture, the most characteristic one is the stalactite growth in bouillon containing oil drops on its surface. The culture grows downward from the under surface of the oil drops as powdery threads. These are very fragile, and as the slightest jar breaks them, it is difficult to obtain this cultural characteristic.

Confusing organisms.—The lesions produced in certain laboratory animals by *B. pseudotuberculosis rodentium* of Pfeiffer and *B. tularense* bear a strong resemblance to those induced by plague, but neither of these is distinctly pathogenic for rats. *B. pseudotuberculosis rodentium* is the only organism morphologically and culturally likely to be mistaken for *B. pestis;* there is even some degree of cross-immunization between these two organisms so that the identity of the two is possible.

Crucial test.—The ability of the plague bacillus to pass through intact shaven skin has been regarded as the crucial test. The weight attached to this test at present is, however somewhat diminished by a similar power on the part of certain other organisms, notably the *B. tularense* mentioned above, which while biologically not closely related to the plague bacillus produces in guinea pigs lesions indistinguishable from those of plague. Organisms other than *B. tularense*, when causing death after inoculation by this method, do not give rise to lesions which might be mistaken for those of plague.

Animal plague.—Mice inoculated at the root of the tail quickly succumb. Rats, this being primarily a disease of rats, are of course susceptible. Other rodents, as squirrels, are susceptible. It has been suggested that a rodent, the Siberian marmot, or tarbagan (*Arctomys bobac*) might be the starting point of plague outbreaks. In natural plague of rats, the lesions which establish a diagnosis even without the aid of a microscope are dark red, subcutaneous injection of the flaps of the abdominal walls as they are turned back, fluid in the pleural cavities, oedematous haemorrhagic periglandular infiltration and swelling of the neck glands, and a yellowish appearance of the liver, which is liberally sprinkled with discrete yellowish-white granules about the size of a pin's head. Less frequently the color of the organ is approximately normal but the surface presents a large number of minute, grayish-white spots which give the organ a stippled appearance as if dusted over with pepper.

The bacillus known as Danysz virus (a bacterial culture used to exterminate rats during a plague epidemic) also causes whitish granules of liver but these are larger and do not have the appearance as if peppered on the liver.

The neck glands in rat plague are chiefly involved because the flea prefers to inhabit the skin of the neck. The spleen is swollen, congested and granular and smears from this viscus will show the bacilli.

A chronic form of rat plague, which may be a factor in keeping up the disease, is characterized by enlargement of the spleen and the presence within it of nodules containing plague bacilli. McCoy has noted that the frequency of the cervical bubo in rats, reported by the Indian Commission as 72%, was not found in California. The glands show distinct caseation with haemorrhage and haemorrhagic periglandular infiltration and injection, as well as enlargement.

Transmission.—Investigations in India definitely determined the fact that the flea *Xenopsylla cheopis* (*X. pallida?*) is the intermediary in the transmission of plague from rat to rat and from rat to man.

In Europe and U.S., *Ceratophyllus fasciatus* is the common rat flea and it, as well as other species of flea, may transmit the disease. The bedbug will also transmit plague. Fleas suck up the septicaemic blood of infected rats and there is a development of plague bacilli in the oesophagus with more or less obstruction. When feeding, such fleas regurgitate and thus inoculate these plague bacilli. The faeces of such fleas are also infectious.

Pneumonic plague.—The infectivity of primary pneumonic plague is very great, and infection seems to be acquired by the respiratory atrium (from man to man). This was the terrifying type of plague in the black death of the fourteenth century.

Strong and Teague have shown that of 39 plates exposed before the mouths of patients with pneumonic plague, with marked dyspnoea and pulmonary oedema, only 1 plate showed plague bacilli when coughing did not take place. In 39 other experimental plate cultures, with coughing on the part of the patients allowed, there were 15 plates showing plague bacilli. The droplet method of infection is therefore the important one in plague pneumonia. As these droplets are expelled to a considerable distance not only should the respiratory inlets of those exposed be protected by masks but the conjunctivae with glasses and abrasions with protective coatings.

For diagnosis make smears and cultures from material drawn from a bubo by a syringe. (At a later stage, when softening begins, there may not be any bacilli present.) Also, if pneumonic plague, make smears from the sputum. Blood cultures and even blood smears may be employed in septicaemic plague. Formol fuchsin and Archibald's stain make satisfactory stains. Always inoculate a guinea pig with the material either by rubbing it in with a glass spatula on the shaven skin or by inserting a piece of the suspected plague tissue into a pocket made by cutting the skin with scissors and extending the wound subcutaneously with scissors or forceps. This is more practical than injecting an emulsion with hypodermic syringe.

For prophylaxis the most important method is that of Haffkine. Stalactite bouillon cultures of plague are grown for 5 to 6 weeks. These are killed by a temperature of 65°C. for one hour. Lysol (0.25%) is added to the preparation and from 0.5 to 4 cc. injected, according to the age and size of the individual treated. Susceptibility is reduced about one-fourth, and of those attacked after previous vaccination the mortality is only about one-fourth of what it is among the noninoculated. Strong prepares a prophylactic vaccine from living plague cultures rendered avirulent. Yersin's serum, made by injecting horses with dead plague cultures and afterward with living ones, is of value prophylactically and has possibly considerable curative power.

The Intestinal Group of Bacilli

By means of cultural characteristics in litmus milk and sugar bouillons we can divide the organisms related to the typhoid bacillus at one extreme, and to the colon bacillus at the other, into three groups.

These bacilli are not only of importance by reason of their pathogenic effect but also because they present problems in their separation which demand the utilization of immunological as well as cultural methods. These bacteria agree in being Gram-negative, nonspore-bearing and nonliquefiers of gelatin. They often have a plump, oval morphology instead of the typical rod shape and frequently show group agglutination.

1. The Eberthella or Typhoid-dysentery Group.—There are three important pathogens in this group: The *B. typhosus*, the *B. dysenteriae*, and the *B. paradysenteriae*. The color of litmus milk is practically unaltered and there is no gas production in either glucose or lactose bouillon. No coagulation of milk. No reduction of neutral red. *B. typhosus* is actively motile, while the *B. dysenteriae* is nonmotile.

2. The Salmonella or Paratyphoid-enteritidis Group.—Besides organisms important for animals and probably at times for man, such as *B. suipestifer*, *B. psittacosis* and *B. icteroides* (interesting historically as having been reported as the cause of yellow fever by Sanarelli), we have three well known pathogens: (1) *B. enteritidis* (Gärtner's bacillus); (2) *B. paratyphosus A.* and (3) *B. paratyphosus B.* In this connection it may be stated that the present view is that hog cholera is caused by an ultramicroscopic organism and not by *B. suipestifer* (hog cholera bacillus).

These organisms cannot be separated culturally, but only by immunity reactions. They do not turn litmus milk pink. They produce gas in glucose bouillon, but not in lactose. They very powerfully reduce neutral red with the production of a yellowish fluorescence. They do not coagulate milk. Litmus milk shows a transient acidity but shortly afterward becomes alkaline, the lilac-blue color being intensified. These organisms are motile.

3. The Escherichia or Colon Group.—These turn litmus milk pink, coagulate milk, reduce neutral red, and show varying degrees of motility. Gas is formed in both glucose and lactose bouillon.

The three groups of organisms just described are nonliquefiers of gelatin. Two intestinal organisms, the *B. cloacae* and the *Proteus vulgaris*, differ in liquefying gelatin.

The Typhoid Group

Bacillus typhosus (*Eberthella typhi*) Eberth, 1880; Gaffky, 1884.—This organism may be isolated from the stools, urine, and the blood of typhoid patients.

At postmortem it can best be isolated from the spleen, but may be present also in pure culture in Peyer's patches which have not ulcerated. When ulceration has occurred contamination with *B. coli* is almost sure. Cultures may be obtained from the liver also. In sections made from spleen the Gram-negative bacilli are apt to be decolorized in the steps of dehydration and clearing. By the use of thionin, immediately blotting and clearing in oil or xylol, clumps of bacilli lying between the cells may be shown.

Morphology and cultural characteristics.—The typhoid bacillus, somewhat more slender than the colon bacillus, is from 1 to 3μ in length and about .6μ in breadth.

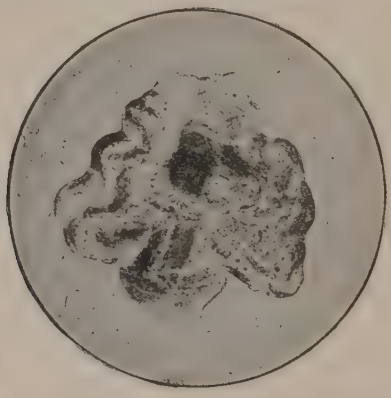


Fig. 43.—Seventy-two-hour-old culture of typhoid bacillus on gelatin. (*Kolle and Wassermann.*)

It is much more actively motile than members of the colon group. It grows readily in bouillon, giving rise to a diffuse clouding. It does not produce indol and does not coagulate milk.

Formerly it was supposed that by the differences in the thickness of the film of a colony or by its varying shades of grayish-blue we possessed data of importance in differentiating typhoid from related organisms. The colonies look like grapevine leaves.

Growth on potato was also considered as affording information. At present, the biochemical reactions give us information assisting in differentiation, and the agglutination and bacteriolytic phenomena, the final diagnosis. The various plating media are considered under media for plating out faeces. See p. 46.

Pathology.—Not only do we find hyperplasia of the endothelial cells in the lymphoid tissue of Peyer's patches and the mesenteric glands and the spleen, with subsequent necroses, but focal necroses of the same character are found in the liver.

A striking feature of the pathology of typhoid fever is the long-continued persistence of the organisms in the gall bladder and elsewhere.

It is believed that a previous typhoid infection, possibly so mild as to have passed unnoticed, is frequently the cause of gall-bladder disease and resulting gall-stones. Various bone infections, especially osteomyelitis, have shown the typhoid bacilli in pure culture.

Lymphatic invasion.—The present view is that typhoid bacilli effect an entrance into the blood stream through some lymphoid channel, as by tonsil or other alimentary lymphoid structure, and develop in the general lymphatic system, the spleen in particular.

Bacteriaemia.—After a time, however, approximately the period of incubation, they become so abundant in these lymphatic organs that they are carried over into the general circulation. Then as a result of bacteriolysis the intracellular toxins are liberated and symptoms develop. If bacteriolysis takes place elsewhere than in the blood we have various suppurative processes. As a result of the formation of antibodies, the development in spleen, etc., is checked; but should these immunity reactions become less potent relapses may occur or various local infections manifest themselves.

As the bacilli do not multiply to any extent in the blood itself the disease cannot be considered as a typical septicaemia but as a bacteriaemia.

Animal susceptibility.—Animals are not susceptible to typhoid fever with the possible exception of the higher apes (chimpanzee). Of course the injection of living or dead cultures may kill an animal but there are no characteristic localizing symptoms.

The blood.—Typhoid bacilli can be isolated from the blood during the latter period of incubation and up to the seventh day of the disease. It is a practical point that the time to isolate the bacteria from the blood is in the first days of the attack. *B. typhosus* appears in the blood in relapses. The diagnosis by agglutination is expected only after the seventh to tenth day. Agglutination may not appear until during convalescence, and fails to develop in about 5% of the cases. It, as a rule, disappears within a year. Before attaching diagnostic weight to a positive agglutination, one should inquire as to recent typhoid vaccination and make quantitative agglutination tests at frequent intervals. An increase in the agglutination titre is usually found after the second week.

Typhoid bacilli may be found not only in the blood, urine and faeces but as well in the sputum of cases showing pulmonary involvement. They have also been found in the cerebrospinal fluid of cases showing meningeal symptoms. The organ-

isms can almost always be recovered from the rose spots, where they exist in the lymph spaces. At autopsy, they may be found in the spleen, Peyer's patches, mesenteric glands and liver.

Curative sera.—Very little success has been obtained with curative sera. Chantamesse, by treating horses with a filtrate from cultures of typhoid bacilli on splenic pulp and human defibrinated blood, claimed to have obtained a curative serum possessing antitoxic power.

Prophylactic vaccination against typhoid.—Wright's method of prophylactic inoculation is now being employed in the British army with success. In this, twenty-four to forty-eight-hour-old cultures are killed at 53°C.; 0.25% of lysol

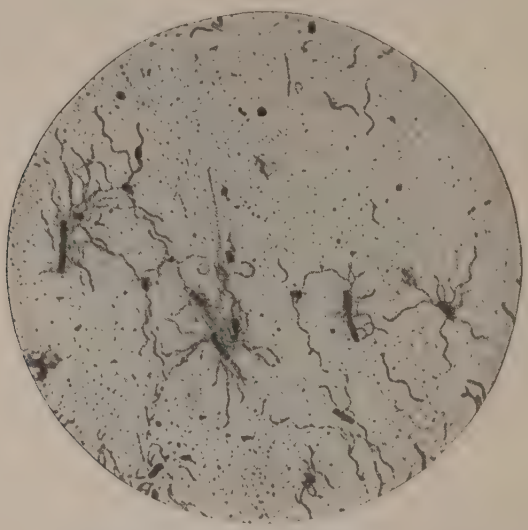


FIG. 44.—Bacillus of typhoid fever, stained by Löffler's method to show flagella. (×1000.) (*Williams.*)

is then added. An injection of 500,000,000 bacteria is made at the first inoculation, and ten days later an injection of 1,000,000,000. The British prefer to inject subcutaneously in the infraclavicular region and at the insertion of the deltoid. The Germans consider 3 injections as conferring greater immunity.

Russell obtained splendid results in the U. S. Army with his method of vaccination. In this, 3 injections were given at intervals of ten days, the dosage being 500,000,000 for the first and 1,000,000,000 for each of the 2 succeeding injections.

On account of paratyphoid fevers appearing in the forces on the Mexican border, vaccination with paratyphoid bacilli was instituted, but separate from the typhoid one.

At present all men in the U. S. Army are vaccinated with a *triple vaccine* made up of 500,000,000 typhoid bacilli, 250,000,000 paratyphoid

A bacilli and 250,000,000 paratyphoid B organisms in 1 cc. of the vaccine.

The first dose is 0.5 cc., the second and third 1 cc. each. The triple vaccine is inoculated subcutaneously at intervals of one week. Craig has carefully studied the reactions following the inoculations of the triple vaccine and could observe no more severe reactions than occur with the typhoid vaccine alone. A temperature rise (rarely above 101°F.) following inoculation is noted in about 32% following the first injection, 86% after the second and 18% after the third. About one-half of those inoculated complain of headache after the first and second inoculations but rarely experience it following the third injection. Malaise is frequently noted.

When tested for agglutinins, one month from the time of the first inoculation, the serum agglutinates typhoid bacilli in from 1–640 to 1–1260 on the average; for paratyphoid A a slightly higher effect, and somewhat less for paratyphoid B. The English, however, obtained higher titres for paratyphoid B than for paratyphoid A. It is usually considered that immunity for typhoid lasts about two years and a degree of protection even after four or five years. With the paratyphoids the immunity is thought to be of less duration. In times of trench warfare it is probably advisable to repeat the inoculations yearly, but ordinarily this may be delayed for three years.

The U. S. Naval personnel receive two courses of three inoculations at intervals of 7 to 10 days, four years apart, of a vaccine containing approximately one billion typhoid bacilli per cc. The first dose is 0.5 cc., the second and third 1 cc. each.

From 1917 to 1924 triple vaccine was used in the Navy. Good statistical evidence showed that paratyphoid infections, other than cases of so-called food poisoning, were rare among naval personnel before paratyphoid vaccine was used. With the vaccine containing *B. typhosus* alone a larger amount of antigen can be employed without undue risk of severe reactions with presumably greater protection against typhoid fever which is the principal infection against which prophylaxis is required.

Carriers.—A very important discovery is that certain persons, who may have had only a slight febrile attack, may eliminate typhoid bacilli for years in their faeces (typhoid carriers). The bacilli are also eliminated for considerable periods in the urine. Distinction is now being made between acute carriers (convalescents) and chronic carriers.

In experiments on higher apes there was evidence that the bacilli eliminated by carriers are in many instances nonpathogenic. About one-half of typhoid cases are believed to be due to contact infections. Drigalski gives it for Germany as 64.7%. The water transmission factor is of less importance than was formerly stated.

The most satisfactory method of detecting carriers is by examination of faeces or urine plated out on Endo's medium. While carriers usually give a Widal reaction this is by no means constant. Typhoid carriers are said to maintain a high opsonic index.

The urine and faeces of typhoid convalescents should be proven negative by cultural procedure before discharging the patients.

Various methods have been tried to cure these carriers, such as vaccination and even cholecystectomy but without any particular evidence of success.

For laboratory diagnosis, blood cultures during the first week and agglutination tests during the second week and onward are the practical methods.

Along with the agglutination tests the urine and faeces should be cultured on Endo's plating medium and later transferred to Russell's medium for cultural identification. The positive identification, provided the culture so isolated shows the cultural characteristics of typhoid, is made by testing the bacilli for agglutination with a known typhoid serum. Instead of the usual blood cultures one may use the clot in the Wright U-tube for culturing and the serum remaining after centrifugalization for the Widal test (*clot culture*).

Mode of infection.—Contact infection is the great factor in perpetuating typhoid fever but nevertheless cases diminish in number each year provided water and milk supplies are safe. The leading European cities as a result of a safe water supply rarely show more than about 3 typhoid deaths per 100,000 population per year. Edinburgh shows less than one per 200,000 for the year 1910. In American cities rates of 12 to 15 per 100,000 were common a few years ago.

Typhoid bacilli have been isolated from water supplies with extreme rarity. The life of the bacillus in the water of streams is a very short one and even in those fulminating outbreaks, from contamination of a water supply, the search for the bacilli is usually unsuccessful. Chlorination is now generally practised and makes for a safe water. In 1922, U. S. death rates fell to 3.15 per 100,000.

Kayser considered that about 27% of cases of typhoid in Strasburg were caused by raw milk, 17% by contaminated water, 17% by contact with typhoid, and 10% were due to typhoid carriers. Other cases were due to infected food and about 13% were of origin impossible to determine. These latter may have been due to unrecognized typhoid carriers. He does not attach the same importance to fly dissemination as do American authors.

Bacillus dysenteriae (*Eberthella dysenteriae*) Shiga, 1898.—Dysentery bacilli produce a coagulation necrosis of the mucous membrane of the large intestine and occasionally of the lower part of the ileum. They are very rarely found in the blood and hence are not eliminated in the urine, but they do occur in mesenteric glands. In general, their distribution corresponds to that of the lesions. Polymorphonuclears are contained in the fibrin exudate.

The toxic products of the bacilli are undoubtedly selective in their action as it has been shown by Flexner that characteristic intestinal lesions may be produced by injecting rabbits intravenously with but a toxic autolysate. The toxin withstands temperature of 70°C. without being destroyed. The toxin may cause joint trouble.

Morphology and cultural characteristics.—The dysentery bacilli as a rule occur singly or in pairs. They are of rather oval or even coccoid shape and somewhat plumper than typhoid bacilli. They may stain bipolarly and are Gram-negative. In all cultural respects the dysentery bacillus resembles the typhoid, and the only practical method of distinguishing these two organisms, other than by agglutination reactions, is by the nonmotility of the dysentery bacillus. The colonies are much like those of typhoid. The paradysentery (Hiss, Flexner and Strong types) colonies often show indentation while the Shiga types show round colonies. The dysentery bacilli do not form those threads or whip-like filaments so characteristic of typhoid cultures.

Types.—There are two main types of dysentery bacilli:

1. Those producing acid in mannite media—the acid strains (Hiss-Flexner-Strong).

2. Those not developing acid in mannite (Shiga-Kruse). Ohno finds that fermentative reactions do not correspond to immunity ones. Thus an acid strain used to immunize a horse may produce a serum more specific for a nonacid strain. Hiss, however, found that organisms similar in fermentation reactions agreed in agglutination ones. The Shiga type is very toxic in cultures, possibly having a soluble toxin, while the Flexner type does not seem to possess a soluble toxin.

Clinically the toxaemia of cases of dysentery due to Shiga types is marked while that from paradysentery strains is slight.

A strain which ferments not only mannite, dextrose, maltose and saccharose, but dextrin as well, is known as the Harris type. An organism resembling the Shiga bacillus but producing indol has been designated the Schmitz bacillus.

The Shiga strains are apt to cause a paresis of the hind extremities of an injected rabbit which may be followed by paralysis and death. At the Lister Institute injections of a soluble toxin produced a serum of marked antitoxic power. Such a dysentery serum, which is probably both antitoxic and antimicrobic, is of curative value. Shiga immunized horses with polyvalent cultures and obtained a polyvalent serum which has reduced the death rate about one-third.

Dysenteric stools.—The appearance of the stool in acute bacillary dysentery is characteristic. There is usually absence of faecal material, and the bulk of the stool is composed of clear, or turbid, amber-colored serous fluid, in which float masses of white mucus showing an arrangement or pattern suggestive of a curly grain in wood, and flecked with

bright red blood. The stained smear is rich in leukocytes, erythrocytes and large phagocytic cells, many of them packed with Gram-negative bacilli.

The bacillus is present in the stools during the first five or six days of the disease. By the tenth day it has probably disappeared. Lactose litmus agar is the most satisfactory plating medium, as it does not restrain growth. The Teague medium is a good one. The stool of the first two days may give practically a pure culture.

Agglutination.—In dysentery patients agglutination phenomena do not show themselves until about the twelfth day from the onset. Hence, this procedure is of no particular value in diagnosis. It is of value, however, to identify an organism isolated from the stools at the commencement of the attack, using serum from an immunized animal or a human convalescent for the agglutination test. Butler has suggested taking serum from dysentery convalescents, noting the strain involved, and preserving it by taking up with filter paper as recommended by Noguchi for the Wassermann haemolytic amboceptor. This I consider very valuable as it is very difficult to immunize rabbits with a Shiga strain on account of its great toxicity. Dean has reduced the toxicity of Shiga vaccines by treating them with eusol.

There seems to be very little agglutinating power in the serum of convalescents from Shiga strains. Flexner strains give agglutination, but early in convalescence the serum is not apt to have a titre of more than 1 to 50. It is usual to accept an agglutination in 1 to 50 dilution as evidence of Shiga infection, and one of 1 to 150 as indicating a Flexner infection.

Ritchie tested the sera of 792 normal persons and found that 30% of these individuals agglutinated Shiga bacilli in 1 to 32, while with Flexner strains 41% agglutinated in 1 to 64 and 30% in 1 to 128. For comparison Ritchie's results with typhoid showed that only 6% agglutinated such bacilli in 1 to 16. There is some evidence that typhoid vaccination increases the agglutinating power of the serum against dysentery organisms. These findings are remarkable, as the usual advice is to consider an agglutination of 1 to 30 as fairly specific for Shiga infections and 1 to 100 for Flexner ones. Ritchie's findings would certainly indicate that a large percentage of normal individuals were at some period carriers of the organism specific for their agglutinating serum. As opposed to this we have a report by Schorer of the most exacting bacteriological and serological examination of organisms isolated from the stools of 1000 soldiers returning from France. Very high titre sera were used in examining suspicious colonies (typhoid serum, 1–32,000, paratyphoid, 1–16,000 and dysentery, 1–4000) using a 1 to 100 dilution for the test (macroscopic). Only one Y type and one Flexner type of *B. dysenteriae* were obtained in the 1000 stools examined.

Ekiri.—In Japan, dysentery-like epidemics of a very fatal disease, termed *ekiri*, occur among young children. The organism is very motile, producing gas and acid in glucose but not in lactose media. It

is reported at times to show indol production. Apparently a member of the Gärtner group.

A strain of dysentery bacilli, known as *Type Y*, has been considered of importance. This organism is very closely related to the Flexner strain and differs from it only in that it requires about forty-eight hours to turn mannite litmus media pink and that maltose litmus remains blue. An organism showing similar cultural characteristics has been recently recovered from faeces of laboratory rabbits by German workers investigating the problem of whether certain animals might serve as carriers for dysentery.

Bacillus fecalis alkaligines (*Alcaligines fecalis*).—This member of the alcaligines group is a frequent inhabitant of the intestinal tract. It does not ferment any of the sugars, and litmus milk cultures become progressively more blue. It is strikingly aerobic.

In milk cultures there is a progressive alkalinity with the liberation of a little ammonia. The organism does not seem to have effect on animals inoculated with it.

It has been isolated from the blood of a few cases which resembled typhoid and has agglutinated (1–50) with the sera of such cases, which did not show agglutination for typhoid. It has also been under suspicion in some cases of diarrhoea of children. Shearman noted the isolation of *B. fecalis alkaligines* from the blood in suspected typhoid cases. These cases showed a sudden onset with slight chill, severe headache, nausea, and occasionally vomiting, with aching of the limbs. The fever would last 2 to 5 days, ranging from 101° to 102°F with often a second pyrexial period.

Salmonella or Paratyphoid—Enteritidis Group

Under this designation may be considered the organisms which cause gastrointestinal disorders of varying degrees. The paratyphoid, bacilli are the most important for man, since, while usually the cause of a disease resembling typhoid, they may be responsible also for gastrointestinal upsets similar to those produced by the Gärtner bacillus. Infection with some members of this group is usually brought about by the ingestion of meat obtained from diseased cattle, the bacilli not being killed unless the meat is thoroughly cooked.

In this group may be placed *B. enteritidis*, the typical meat-poisoning organism *B. paratyphosus B*, *B. danysz*, *B. aertryck*, *B. typhi murium* and *B. suipestifer*.

B. suipestifer or the hog cholera bacillus was formerly thought to be the cause of this important epizootic. It is found in the intestines of quite a percentage of healthy hogs. The cause is now known to be a filterable virus.

These organisms are all alike morphologically and culturally and show quite a tendency to bipolar staining and reduction of neutral red with fluorescence in forty-eight hours. *B. paratyphosus B*, *B. aertryck* and *B. suipestifer* are alike also from an agglutination standpoint, while *B. enteritidis* and *B. danysz* show similarity in this respect. *B. paratyphosus A* stands by itself.

Bacillus enteritidis (*Salmonella enteritidis*) Gärtner, 1888.—This organism has been frequently isolated from cases of gastroenteritis arising from ingestion of infected meat.

Meat from healthy animals which has been in contact with that of diseased animals may become infected. The simple act of placing a piece of infected meat on a sound piece may infect the latter. It has been noted that the bacteria, or their toxins, may be distributed unevenly in the meat eaten, so that one person may be made very ill while others may escape infection. Infection of food may occur not only from unclean handling but from the material carried by flies or even from the faeces of mice or rats deposited on foodstuffs.

This organism is very pathogenic for laboratory animals, producing a haemorrhagic enteritis and at times a septicaemia. Where meat has been contaminated with Gärtner's bacillus, toxins may have been produced, and symptoms of poisoning with acute gastroenteritis would then occur shortly after ingestion. This poison is not a true toxin as it does not require a period of incubation before manifesting its toxic action. It is interesting to note that it is not destroyed by boiling temperature, thus differing from the toxin of the other important meat-poisoning bacillus—*B. botulinus*—which is rendered innocuous by a temperature of 70° or 80°C. If there is only a little toxin introduced with the contaminated meat, the symptoms will be delayed one or two days. *B. enteritidis* has been isolated in pure culture from cases with high fever, marked intestinal derangement, with considerable blood in the rather fluid stools. In two cases studied the disease was at first diagnosed as a severe typhoid infection. Klein thinks the organism of Danysz's virus (used to kill rats during plague epidemics) may be identical with *B. enteritidis*.

Ptomaine poisoning.—It may be stated that such a diagnosis is practically never the correct one. The split products of protein putrefaction have not been demonstrated to have toxic effect when ingested. Such diagnoses are often applied to cases which are found to be from "enteritidis," paratyphoid, dysentery or colon infections. Rosenau has stated that after investigating cases of ptomaine poisoning the explanation was to be found in many different causes. He cites oxalic acid poisoning from eating cooked rhubarb leaves or from sour-grass. Sour-grass soup showed 10 grains of oxalic acid to the pint. Uraemia is also to be considered. Botulism must also be kept in mind. Anaphylactic reactions to certain food stuffs are well known, especially urticarial rashes following shell-fish ingestion. The anaphylactic manifestations may produce their effect in the alimentary tract mucous membranes and be mistaken for ptomaine poisoning.

Burke and May have tabulated the differences in botulism and food infections as follows:

	Botulism	Food infections
Cause	Botulinus toxin	Bacilli of the paratyphoid-enteritidis group.
Fever	Not characteristic; temperature usually subnormal.	Characteristic; acute.
Occurring	Mainly in winter.	Mainly in summer.
Associated with	Preserved foods.	Fresh foods, or freshly contaminated foods, usually meat or milk.
Condition of bowels	Constipation; rarely diarrhoea.	Diarrhoea; offensive.
Visual disturbances	Double vision; ptosis of lids.	Absent.
Abdominal pain	Absent.	Present.
Onset	Usually gradual.	Sudden.
Incubation period	Variable, usually from twelve hours to several days.	Short, usually from six to twelve hours.
Throat	Swallowing difficult.	Normal.
Treatment	Antitoxin.	Systemic.
Mortality	From 60 to 70%	From 1 to 2%

Paratyphoid Bacilli (Achard and Bensaude, 1896; Schottmüller, 1901).—Cases resembling mild attacks of typhoid occasionally show agglutination for paratyphoid bacilli. These organisms have been isolated from the blood, as with typhoid. Two types have been recognized, the paratyphoid A (*Salmonella paratyphi*), and the paratyphoid B (*Salmonella schottmülleri*), the latter occurring in 80% of paratyphoid infections. Culturally, paratyphoid B cannot be separated from Gärtner's bacillus. With paratyphoid A there is less gas produced in glucose bouillon than with paratyphoid B, and the primary acidity of litmus milk is not succeeded by a subsequent alkalinity. It does not seem practical to draw a fine distinction between these two strains.

Krumwiede attaches importance to acid production in xylose by all members of the Gärtner group, except *B. paratyphosus A*.

The brownish discoloration on lead acetate agar by *B. paratyphosus B* is also a differentiating point.

The same points as to transmission, diagnosis and carrier state noted under typhoid hold for the paratyphoids.

The symptoms of paratyphoid A infections are rather like those of typhoid but generally milder. Cases have been reported where paratyphoid A even showed the intestinal lesions of typhoid. Various local infections with the paratyphoids, especially pyelitis, have been noted. Some cases of infectious jaundice have been attributed to paratyphoid infection.

Paratyphoid B not only gives symptoms resembling a mild typhoid infection, but may show symptoms more like those of meat-poisoning or even cholerine. It is more pathogenic for laboratory animals than is *B. typhosus*. The development of antibodies upon immunizing a man or animal with paratyphoid organisms does not seem to approach that obtained with typhoid.

Castellani has conducted experiments with typhoid and paratyphoid vaccines and has found that typhoid vaccines give an agglutinating serum of about 1 to 350 titre from the second to fifth week dropping to about 1 to 100 after three or four months. Paratyphoid A gives one of about 1 to 75 for the first month which drops to about 1 to 60 after four months. Paratyphoid B gives about one-half the agglutination response that paratyphoid A does. These results do not correspond with those of Craig noted under typhoid vaccination.

A critical study by Miller brings out the following points: During the war paratyphoid A cases would be at one time more common and at another time paratyphoid B ones. The paratyphoid organisms may give rise to as serious manifestations as typhoid, which was particularly true of typhoid in the inoculated. As a result of the paratyphoid bacilli having a greater tendency to generalize there is less of the localization in the lymphatic tissues of the intestine than with typhoid. As a result many types of paratyphoid are noted. Among *typhoid types* the general manifestations greatly resemble typhoid but haemorrhage and perforation are much less frequent. The *dysenteric type* may be clinically dysentery or it may be associated with general symptoms. The *nephritic type* is rather frequently noted; this may be of the nature of nephritis or pyelitis. There is also a *rheumatic type* and an *influenzal* one and in this latter type we may have a respiratory, gastrointestinal or nervous grouping. Miller notes that the infectivity of paratyphoid is very great.

B. morgani (*Salmonella morgani*).—Morgan has reported as the cause of certain cases of bacillary dysentery a bacillus known as *B. morgani*. It is motile, produces indol, and in glucose bouillon gives a very slight amount of gas.

It does not change mannite and does not produce a primary acidity in litmus milk. This organism is a frequent cause in England of summer diarrhoea of children. Flies from houses with such cases often show Morgan's bacillus. A dysentery type much like the Flexner-Strong strain is often found in the enteric affections of children in the United States.

Escherichia or Colon Group

B. coli (*Escherichia coli*).—The most important member of this group is *B. coli*, an organism reported by Escherich, in 1886, as present in the faeces of infants. In plating from the faeces of normal adults it is almost always present among the colonies developing. Its presence in water is an index of sewage contamination. It is an oval to rod-shaped organism and averages about .6 × 2.5μ in size. Even in the bacillary forms the ends are rounded. While on the whole a motile organism, the degree of motility varies, and in the same culture motile and nonmotile forms are observed. Young cultures tend to show greater motility, which is often lost as the culture becomes older. It is Gram-negative, produces indol and acidifies and coagulates litmus milk. It grows readily and luxuriantly on all ordinary culture media and in bouillon gives rise to uniform turbidity, with at times a surface scum and often a somewhat foetid odor. Gelatin is not liquefied. It ferments glucose and lactose with gas production.

B. coli communior (*Escherichia communior*).—This closely related organism, which was given its name from its supposed greater frequency in faeces, is similar in all respects to *B. coli* except that it gives gas in saccharose bouillon, which property is not possessed by the colon bacillus. This bacillus as well as the colon bacillus ferments dulcite.

B. aerogenes (*Aerobacter aerogenes*).—This organism, like *B. coli communior*, produces gas in glucose, lactose and saccharose bouillon, but does not ferment dulcite. It is as a rule distinctly nonmotile and often seems to have capsular material surrounding it. It is almost constantly found in the upper intestinal tract of nursing infants and is an important organism in the souring of milk. Except for its greater fermentative activities it may be readily confused with the Friedländer bacillus. *B. aerogenes* grows readily on all media and on potato slants the culture tends to show gas bubbles.

Organisms of the colon group are not confined to human faeces but are found in the intestinal tracts of other animals. Organisms apparently similar may be obtained also from various cereals, it being probable, however, that they are derived from the faeces of rats feeding on such grains.

Some consider that the *B. coli* produces a bactericidal substance which inhibits the growth of, or destroys, pathogenic bacteria which may have escaped the destructive influences of the gastric juice; others that this effect is due to their free growth and the development of phenol and various putrefactive substances.

The probable importance of the colon bacillus in protecting the organism is shown by the fact that where numerous colonies of pathogenic organisms may be cultivated from faeces we may find absence of the colon bacilli or a diminution in their number. This condition may be observed in infections with the organisms of dysentery, cholera, typhoid and paratyphoid. While its normal function is probably protective, yet the *B. coli* is an important pathogenic agent, it being particularly prone to cause lesions of the bladder and pelvis of the kidney, and having at times been the only organism isolated from purulent conditions within the abdominal cavity, especially in appendicitis and lesions about the bile ducts. In young children, a colon bacillus pyelitis is not infrequently a cause of fever. Normal serum agglutinates the colon bacillus in dilutions of 1–10 or 1–20 and this is increased following enteric infections (group agglutination). Owing to the tendency of the colon bacillus to post-mortem or agonal invasion of blood stream or organs we must be very conservative in accepting it as explaining infections. Members of the colon group are more apt to be secondary to primary infections by other more distinctly pathogenic bacteria such as the ordinary pus organisms. The colon bacillus has a wider pathogenic rôle in the tropics than in temperate climates and many cases having a prolonged fever course may show a colon bacteriaemia. In cases going on to a fatal termination abscess formation in the kidneys is apt to be present. At times indolent skin lesions, resembling furunculosis, may be of colon origin.

B. aerogenes is considered as having less pathogenic effect than *B. coli*. It may cause a cystitis and in such case may be associated with pneumaturia.

B. coli is fully considered under water bacteriology.

Gelatin-liquefying Faecal Bacteria Group

There is a group of putrefactive organisms usually designated as the proteus group which would appear to have pathogenic effect somewhat similar to that of *B. coli*.

The gelatin-liquefier, *B. cloacae*, is closely related to *B. coli* and is of interest in water bacteriology.

Proteus vulgaris.—This organism is often encountered in plates made from faeces, or sewage-contaminated water.

It is common in decaying meat or cheese, and cases of even fatal poisoning with marked gastrointestinal symptoms and cardiac failure have been reported. Such poisoning may possibly have been due to ptomaines elaborated by the proteolytic action of the organism on the food, rather than from *B. proteus* itself. At times it is the cause of cystitis. The colonies on agar are moist and unevenly spreading (amoeboid). The bacillus is very motile, long and slender, tends to form filaments and, as a rule, is Gram-negative. It digests blood serum and is a rapid liquefier of gelatin. In litmus milk it coagulates with a soft clot and an alkaline reaction. Subsequently the litmus is reduced and the clot digested, giving a dirty yellowish-brown fluid. Indol is rarely produced. The cultures generally have a putrefactive odor. In infective jaundice (Weil's disease) this organism has been reported as the cause. Organisms of this group were formerly designated as *B. termo*.

Weil-Felix reaction.—Certain organisms of the proteus group have recently come into prominence in the diagnosis of typhus fever. These bacilli, designated X_2 and X_{19}, show agglutination with the sera of most typhus fever cases. This test (Weil-Felix reaction) is described under typhus fever. These strains correspond in characteristics to certain strains of *Proteus vulgaris*, producing indol in peptone solution, and acid and gas in glucose, maltose and saccharose, but not in lactose or mannite. They digest gelatin and blood serum somewhat more slowly than typical cultures of *Proteus vulgaris*. Although these organisms have been isolated from the urine of several typhus cases, it seems certain that these *X* bacilli are neither causative organisms nor secondary invaders. The reaction is therefore heterologous and not specific.

B. cloacae (*Aerobacter cloacae*).—Isolated first from sewage by Jordan. It is, as a rule, a rapid liquefier of gelatin, although not as active as the proteus group, and in its reactions with sugars and litmus milk resembles the colon bacillus.

Where the gelatin liquefaction is slow or slight *B. cloacae* may be distinguished from *B. coli* by its gas formula which is about three times as much CO_2 as H, just the reverse of that of the colon bacillus.

The Group of Lactobacilli

These are often termed the long-rod group of lactic acid bacteria in contradistinction to certain other Gram-positive bacilli which are short and oval and which may be confused with the so-called milk streptococci.

The long-rod group often forms chains and often shows metachromatic granules which stain with Neisser's method. They are readily distinguished from Gram-negative lactic acid-producers, of which the type is *B. aerogenes*, by their Gram-positive staining. *B. acidophilus* often gives the impression of a diphtheroid in a Gram-stained faeces smear. It is nonmotile and often shows polar granules. Grows only at temperatures above 22°C., opt. 40 to 45°C. It grows better anaerobically than aerobically. In anaerobic cultures, it shows the clubbed involution characteristics of *B. bifidus;* so that some consider these organisms the same, the morphology of *B. bifidus* being the result of anaerobiosis. Original cultures are best made in 1% glucose and 1% acetic acid bouillon. Some authorities consider *B. bifidus* the most important representative of the large intestine flora. This organism is usually described as an obligate anaerobe and in anaerobic cultures shows the bifid ends which give it its name, but this characteristic is not found in smears from the faeces of nursing infants. *B. acidophilus* is more common in such smears and this organism is usually described as an aerobe. *B. acidi lactis* is less thermophilic than *B. acidophilus* and coagulates milk which *B. acidophilus* does not do. Certain polar-granule bacteria, as *B. granulosus*, found in Yoghurt, are similar to *B. acidophilus* but coagulate milk; no gas. *B. bulgaricus* is the type of the group and is discussed

under "milk." The "lactobacilli" are able to multiply in the presence of large amounts of acid. They are acid-resisting rather than acidophil. Such type organisms are present at times in faeces. The administration of lactose tends to cause the appearance of *B. acidophilus* in stools. With cultures frequent transfer is necessary as viability decreases rapidly.

Rodella thinks *B. acidophilus*, *B. bifidus*, *B. gastrophilus* and the Boas-Oppler bacillus identical. *B. bulgaricus* is said never to show polar granules. *B. bulgaricus* and the group of organisms similar to it found in buttermilk, etc., are widely used in the treatment of various intestinal troubles. North has used cultures of *B. bulgaricus* for extermination of undesirable organisms in other parts of the body than the alimentary canal (used as applications in nasal, throat or genito-urinary infections). At present we consider the "bulgaricus" group as of no value in mucous membrane infections. Their implantation in the intestinal tract is considered improbable so that we now depend upon change of diet to alter bacterial flora of the intestines. Kendall is of the opinion that *B. acidophilus*, a normal inhabitant of the large intestine, would theoretically be more apt to be of value than *B. bulgaricus*, which can be considered a milk organism and would have to become adapted to the intestinal environment.

Chromogenic Bacilli

These are identified by the color of their colonies on agar. The *B. pyocyaneus* is the most important one of them in medicine, but the *B. prodigiosus* is also of interest. A violet chromogen, the *B. violaceus*, which is motile and liquefies gelatin, has been described under many names. It has been found in water.

An orange-yellow chromogen, the *B. fulvus*, is nonmotile and varies as to its liquefaction of gelatin.

B. pyocyaneus (*Pseudomonas aeruginosa*) Gessard, 1882.—This organism is frequently termed the bacillus of green or blue pus. It is a small ($2.5 \times 0.5\mu$), motile Gram-negative bacillus.

It is generally a slender delicate rod, often showing thread-like arrangement, but at times it may be short and plump. It grows readily at room or body temperature. It liquefies gelatin rapidly. The green color diffuses through the agar or gelatin on which it grows, so that we not only have the green-colored colony, but the medium as well is colored. Upon potato the colonies are more of a deep olive green to dirty brown.

No gas is produced in either glucose or lactose bouillon; blood serum is digested, the pitted surface showing a reddish brown color. The protein ferment pyocyanase has been used to remove diphtheritic membrane and for treatment of nasal catarrhs due to *M. catarrhalis*. There are 2 pigments—a green water-soluble one and a blue one soluble in chloroform.

It is widely distributed in water and air, and is frequently isolated from faeces. The *B. fluorescens liquefaciens* of water seems to be simply a strain of *B. pyocyaneus*. The *B. pyocyaneus* is frequently associated with other pus organisms in abdominal abscesses.

In addition to having an endotoxin, it produces a soluble toxin similar to diphtheria toxin. This toxin differs from those of diphtheria and tetanus in that it can withstand a temperature of 100°C., while those of diphtheria and tetanus are destroyed at about 65°C. The fact that the union between toxin and antitoxin is only of a binding, neutralizing nature is best shown by taking a mixture of pyocyaneus toxin and antitoxin, which is innocuous, and heating it. This destroys the

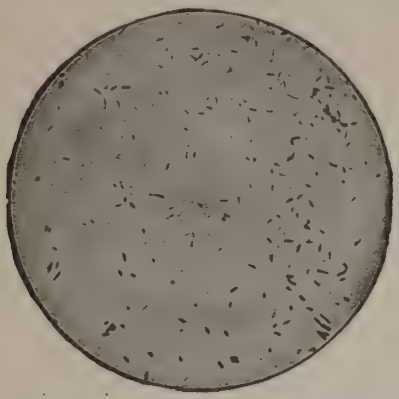


FIG. 45.—*Bacillus pyocyaneus.* (*Kolle and Wassermann.*)

antitoxin, but does not injure the toxin. We now find that the original toxicity has returned. The antitoxins of diphtheria and tetanus are more stable than the corresponding toxins; hence, this result would be impossible with them, as upon heating we should first destroy the toxin.

On account of the frequent association of *B. pyocyaneus* with other organisms of better recognized pathogenicity it has been considered rather harmless; this view can no longer be entertained as it is frequently the sole cause of middle-ear inflammations, intestinal disorders, cystitis and possibly at times of septicaemia.

B. prodigiosus (*Serratia marcescens*).—This is a very small coccobacillus which shows motility in young bouillon cultures. It is Gram negative. The colonies on agar or other solid media show a rich red color. The pigment develops only at room temperature, it being absent in cultures taken out of the incubator. The *B. prodigiosus* is frequently found on food-stuffs, especially bread, where it may simulate blood. It liquefies gelatin rapidly and gives a diffuse turbidity to bouillon. It is probable that *B. indicus* and *B. kiliensis* are strains of *B. prodigiosus*.

Coley's fluid, which has been used in cases of inoperable sarcoma and other malignant growths, is a culture prepared by growing very virulent streptococci in bouillon for ten days. This *Streptococcus* culture is then inoculated with *B. prodigiosus*, and after another ten days the mixed culture is killed by heat at 60°C. and the sterile product injected. Coley injected about 0.05 cc. of this vaccine. He used nonfiltered, heat-sterilized bouillon cultures of a *Streptococcus* obtained either from a case of erysipelas or septicaemia. To this is added material from agar cultures of *B. prodigiosus*, grown separately and sterilized before adding to the sterilized streptococcus bouillon culture.

CHAPTER IX

STUDY AND IDENTIFICATION OF BACTERIA—SPIRILLA. KEY AND NOTES

KEY to recognition of gelatin-liquefying, motile and Gram-negative spiral or comma-shaped organisms.

I. Do not give the nitroso-indol reaction (cholera red) with sulphuric acid alone in twenty-four hours, and furthermore, especially in the case of Denecke's spirillum, the cholera red reaction may be negative after prolonged cultivation.

(a) Produce an abundant moist cream-colored growth on potato at room temperature.

(1) Vibrio proteus (Finkler and Prior's spirillum). Liquefaction of gelatin very rapid. No air-bubble appearance at top of liquefied area. Cultures have foul odor. Milk coagulated. Thicker and somewhat larger spirillum than that of cholera. Isolated from cholera nostras.

(b) Scanty growth or none at all on potato at room temperature. Only a moderate yellowish growth when incubated at about body temperature.

(2) Vibrio tyrogenus (*Spirillum tyrogenum;* Denecke's spirillum). Does not liquefy gelatin so rapidly as that of Finkler-Prior. Milk not coagulated. Thinner and smaller spirillum than that of cholera.

II. Give the nitroso-indol reaction with sulphuric acid within twenty-four hours.

(a) Very pathogenic for pigeons.

(1) Vibrio metchnikovi (*Spirillum metchnikovi*). Liquefies gelatin about twice as rapidly as the cholera. Gives bubble appearance at top of stab. Produces an acute enteritis in fowls. Injection of culture into pectoral muscles of pigeons produces a fatal septicaemia. Not pathogenic for man.

(b) Scarcely pathogenic for pigeons.

(2) Vibrio comma (*Spirillum cholerae*).

Note.—Nonmotile, nonliquefying and Gram-positive spirilla have also been described. There is also a large group of phosphorescent spirilla.

Spirillum cholerae (*Vibrio comma*) Koch, 1884. *Morphology.*—Typically, the morphology of this organism is that of the comma (comma bacillus of Koch). It also frequently shows S-shapes, and often appears in long threads showing turns. When freshly isolated from cholera material it, as a rule, shows a fairly typical morphology but, after subcultures in the laboratory, variations are common, so

that rod forms and round involution shapes give a picture altogether at variance with the comma shape.

Even in recent cultures of undoubted cholera we may have different forms, as coccoid forms and slender rods. Ohno has noted the fact that the same strain of

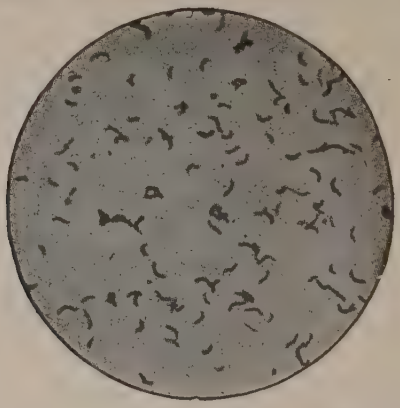


FIG. 46.—Cholera spirilla. (*Kolle and Wassermann.*)

cholera organism will give at one time vibrio forms and again coccoid or rod forms, depending on the reaction of the media. Inasmuch as the recognition of vibrio shapes is of importance in diagnosis he recommends that material from a stool be inoculated into 3 tubes of peptone solution of reaction +0.3, −0.5 and −1, respectively, one of which would probably show vibrio morphology.

FIG. 47.—Involution forms of the spirillum of cholera. (*Van Ermengem.*)

The cholera spirillum is very motile (a scintillating motility) and liquefies gelatin fairly rapidly, although more slowly than any of the spirilla mentioned in the key.

Cultural characteristics.—Colonies on gelatin show in twenty-four hours as small granular white spots which have a spinose periphery. An encircling ring of liquefac-

tion now makes its appearance and the highly refractile (as if fragments of sparkling glass) colony can be separated into a granular center, a striated periphery, and a clear external ring of liquefaction. These characteristics were formerly considered as peculiar to cholera organisms, but they are not now so regarded.

On gelatin stabs the liquefaction produces a turnip-like hollow at the top of the puncture—the air-bubble appearance. It gives the nitroso-indol reaction with sulphuric acid alone (cholera red). Kraus attaches importance to the fact that the cholera spirillum does not produce a haemolytic ring on blood agar as do the pseudocholera spirilla; a difficulty is that many pseudospirilla do not haemolize. Furthermore, true cholera strains may occasionally show haemolysis, especially in laboratory cultures. Quite a discussion has arisen in connection with a spirillum isolated from cases of diarrhoea (no symptoms of cholera) in pilgrims at El Tor. This organism gave the immunity reactions (agglutination) of true cholera but on account of its haemolytic power has been considered as distinct from cholera. Such a view would seem to be untenable. *Sp. choleræ* grows very rapidly in peptone solution and this is the medium for the enrichment test to be later described. On this it may form a pellicle. On agar the colony is more opalescent (more of a translucent grayish-blue) than the typhoid. It does not grow on potato except at body temperature. It does not coagulate or acidulate litmus milk. Some strains, however, do produce a certain amount of acid. Using the Hiss serum sugar media our strains produced acid in glucose and saccharose but not in lactose. No gas production in any of the sugars. The spirilla are found in myriads in the rice-water discharges, so named from the white flakes consisting of desquamated epithelial cells. They penetrate the crypts of Lieberkühn, but rarely extend to the submucosa. The symptoms are due to an endotoxin.

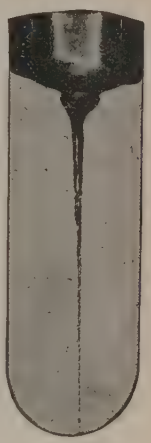


FIG. 48.—Spirillum of cholera. Stab culture in gelatin two days old. (*Fraenkel and Pfeiffer.*)

Transmission.—Cholera may be transmitted by water supplies, when the outbreak is apt to be widespread and involve great numbers from the start; by indirect contagion, as through flies or green vegetables, and—a very important point—by well persons whose faeces contain virulent cholera spirilla (cholera carriers).

Cholera spirilla disappear from the stools of cholera patients very rapidly, usually in five to ten days.

Carriers.—Only exceptionally are organisms excreted longer than three or four weeks, but cases are on record of periods approximating two or three months. Cholera carriers in good health may come down with cholera as the result of administration of purgatives or from alimentary-canal disorder. This would explain periods of incubation longer than the usual one or two to five days.

Cholera carriers are therefore of less importance epidemiologically than typhoid carriers, where the carrier stage may last years.

It is well to remember, however, that cases have been reported of positive findings after a period approximating two months from the onset of the attack of cholera. Another important consideration is that the vibrios may be absent at one examination and be present at a later one. Purgatives seem to influence the reappearance of the spirilla. The maintenance of an acid reaction of the faeces, as is induced by the continued ingestion of lactic acid bacteria, would apparently be of value in the treatment of cholera carriers.

Greig has found infection of the bile of the gall bladder or ducts in 80 cases in 271 cholera autopsies. While cholera spirilla are soon crowded out of the intestine by bacteria, thus explaining the short period during which cholera spirilla are usually excreted by convalescents, this is not true when the cholera vibrio gets into the bile ducts or gall bladder, where ideal conditions prevail for a prolonged life. In fact bile has recently been recommended as a selective medium for cholera enrichment and is to be recommended as the medium in which to inoculate material which cannot be transferred quickly to the laboratory. The spirilla die out in a short time in faeces but will live in bile for several weeks. Greig found one cholera convalescent excreting cholera vibrios forty-four days after the attack. Of 27 persons who had been in contact with cholera patients 6 were excreting cholera vibrios, though apparently well. In small areas of consolidation of the lungs of those developing pneumonia during convalescence Greig obtained cholera vibrios. He was unable to cultivate vibrios from the blood, but in 8 cases out of 30 isolated them from the urine. It has been stated that positive blood cultures have been obtained.

Identification.—To identify cholera spirilla immunity reactions are necessary:

1. Injected intraperitoneally into guinea pigs, it produces a peritonitis and subnormal temperature. This reaction exists for spirilla other than the true cholera spirillum.

2. Intramuscular injections into pigeons are only slightly pathogenic, if at all.

3. The agglutination test is the most practical. In this we use a high-titre serum from an immunized animal, in dilution of from 100 to 1000. It is rare that true cholera vibrios fail to agglutinate in serum of 1 to 500 and even sera of 1 to 10,000 dilution give the reaction. Dunbar's quick method is very practical. Make two hanging-drop preparations, using mucus from the stool as the bacillary emulsion. To one add an equal amount of 1:50 normal serum; to the other a 1:500 dilution of immune serum. Cholera spirilla remain motile in the control, but lose motility and become agglutinated in the preparation with the immune serum.

4. Pfeiffer's phenomenon. If cholera spirilla are introduced into the peritoneal cavity of an immunized guinea pig (or if, together with a 1:1000 dilution of immune serum, the mixture is injected intraperitoneally into normal guinea pigs) and at periods of ten to sixty minutes after injection material is removed by a pipette from the peritoneal cavity, the spirilla will be found to have lost motility, have become granular and degenerated. Pseudospirilla are unchanged. This reaction may be carried on in a pipette, using fresh serum.

Serum treatment.—On the whole the reports from the use of anticholera sera are not very encouraging. Savas, however, was favorably impressed by such treatment during the Balkan war. Serum should be administered intravenously and early in the attack and given in doses of 50 cc. Of 61 severe cases so treated the mortality was 55.7%. Of 17 severe cases not receiving serum treatment all died.

Vaccination.—This method of protection was first practised by Ferran, in 1885, using live cultures. Accidents occurred and such inoculations were stopped. In 1893, Haffkine made preliminary subcutaneous injections of an attenuated living culture which was later followed by injection of living cultures, the virulence of which had been exalted by passage through animals to a fixed degree of virulence. He now uses the highly virulent culture alone. This vaccine is not killed by heat. Besredka, Shiga and Masaki have noted the higher immunity conferred by vaccines sensitized with cholera-immune serum.

It is generally stated that the presence of antibodies following vaccination is of short duration—3 or 4 months, but this is true of the antibody-formation following an attack of cholera, which nevertheless confers a lasting immunity. In a case of cholera, agglutinins appear about the fourth day, to reach a maximum in 10 to 14 days, and diminish after the third week. The serum of convalescents rarely agglutinates in higher dilution than 1 to 200, although in some cases in 1 to 1000.

Strong uses a cholera autolysate. In this, cholera cultures are killed at 60°C. The killed culture is then allowed to digest itself in the incubator at 37°C. for three or four days (peptonization). The preparation is then filtered and from 2 to 5 cc. of the filtrate is injected.

At present the same methods are being used for the preparation of cholera vaccines as for those of typhoid. An emulsion of the vibrios in salt solution or a bouillon culture is subjected to a temperature of 54°C. for one hour. Three doses are injected seven to ten days apart, going from 500,000,000 to 2,000,000,000.

In the Balkan war (1913) Kolle's vaccine was employed with considerable success. This vaccine is killed by exposure to 58°C. for one hour. It was found that this vaccine was not only of value prophylactically but diminished case mortality as well. With 91,224 persons vaccinated and 8968 not vaccinated, the case rate among the inoculated was 0.7% and the death rate 10.2%, while among the noninoculated the case rate was 9.3% and the death rate 27.5%.

Ottolenghi prefers to sterilize with a temperature of 53°C. He gives 500,000,000 at the first injection and 2,000,000,000 at the second.

Among 72,653 soldiers, having two inoculations of this vaccine, the incidence of cholera was about 13 times less than among 14,332 who were not vaccinated.

Of 2897 Greek sanitary corps men inoculated 0.45% were attacked while of 114,805 combatants, not inoculated, about 2% were attacked by cholera. One would naturally consider the greater exposure of the sanitary forces.

Laboratory diagnosis.—1. Take a fleck of mucus, make a straight smear and fix; stain with a 1:10 carbol fuchsin. The comma-shaped organisms appear as fish swimming in a stream.

2. Inoculate a tube of peptone solution. The cholera spirilla grow so rapidly, and being strong aerobes, they grow on the surface of the fluid, so that by taking a loopful from the surface, we may in three to eight hours obtain a pure culture. Should there be a pellicle present, this should be avoided in the transfer by tilting the tube slightly, so that the material near the surface can be obtained without touching the pellicle. Inoculate a second tube from the surface of this first and, if necessary, a third (enrichment method).

3. Test for cholera red reaction done simply by adding from 3 to 5 drops of concentrated chemically pure sulphuric acid to the first or second peptone culture after

eighteen to twenty-four hours' growth. Some specimens of peptone do not give the reaction. At times we only get the cholera red when we have a pure culture of cholera.

4. Smear a fleck of mucus or, better, the three-hour surface growth of a peptone culture on a dry agar surface in a Petri dish. From colonies developing, make agglutination and, if desired, cultural tests. It is by immunity reactions that we identify cholera spirilla. The surface moisture of plates is best dried by the filter-paper top.

The cholera colony is easily distinguished from the ordinary faecal bacterial colonies by its transparent, bluish-gray, delicate character. It emulsifies with the greatest ease. A practical, quick method is to make smears from suspicious colonies, stain for one minute with dilute carbol fuchsin and, if vibrios are present, to make two vaseline rings on a single slide allowing ample space at one end for handling the preparation safely. Inside of one ring deposit with a platinum loop a drop of salt solution and inside the ring nearest the end which is to be held by fingers or forceps, deposit a loopful of 1 to 500 or 1 to 1000 dilution of cholera serum. The emulsion in the salt solution remains uniformly turbid, and under a low power of the microscope (⅔ inch) shows a scintillating motility. The emulsion made into the drop of serum quickly shows a curdy agglutination and upon examination with the ⅔-inch objective shows clumping and absence of motility. Cover glasses placed over the two vaseline rings assist in the study of the preparation.

Cholera selective media are considered under "Culture Media."

CHAPTER X

STUDY AND IDENTIFICATION OF MOULDS

CLASSIFICATION OF THE FUNGI

Class	Order	Family	Genus	Species
Phycomycetes	Mucorales	Mucoraceae	Mucor	M. corymbifer
				M. mucedo
			Rhizomucor	R. parasiticus
			Rhizopus	R. niger
Ascomycetes	Gymnoascales	Saccharomycetaceae	Saccharomyces	S. cerevisiae
				S. anginae
				S. blanchardi
			Endomyces	E. vuillemini
			Cryptococcus	C. gilchristi
			Coccidioides	C. immitis
		Gymnoascaceae	Trichophyton	T. sabouraudi
				T. tonsurans
				T. violaceum
				T. mentagrophytes
			Epidermophyton	E. cruris
			Endodermophyton	E. concentricum
			Montoyella	M. nigra
				M. bodini
			Microsporum	M. audouini
	Sphaeriales	Perisporiaceae	Achorion	A. schoenleini
			Penicillium	P. montoyai
				P. crustaceum
			Sterigmatocystis	S. nidulans
			Allescheria	A. boydii
			Aspergillus	A. fumigatus
				A. pictor
				A. niger
Hyphomycetes			Discomyces	D. bovis
				D. madurae
			Madurella	M. mycetomi
			Malassezia	M. furfur
			Nocardia	N. asteroides
			Microsporoides	M. minutissimus
			Monilia	M. albicans
				M. candida
			Trichosporum	T. giganteum
			Sporotrichum	S. schencki
				S. beurmanni

NOTE.—In many of the works on bacteriology considerable space is given to the so-called Higher Bacteria. The organisms are chiefly considered under the names *Leptothrix* or forms in which are found simple nonbranching threads. *Cladothrix* or thread-like forms with false branching and *Streptothrix* or forms showing true branching. It is not practical to consider any separate group distinct from the so-called Lower Bacteria on the one hand and the Fungi on the other.

General Considerations of Plant-parasites

The vegetable kingdom is divided into the following phyla: Myxophyta or slime moulds, Schizophyta or fission fungi, Thallophyta embracing algae, fungi and lichens, Bryophyta or mosses and liverworts, Pteridophyta or ferns, and the Spermatophyta, the seed plants.

The only botanical phyla which concern us in parasitism of man are the Schizophyta and the Thallophyta.

The classes Schizomycetes and Schizophyceae belong to the phylum Schizophyta. In the class Schizomycetes we have the order of Eubacteriales which embraces such important families as Spirillaceae, Coccaceae, Bacillaceae, etc., which are considered in the preceding chapters. To the class Schizophyceae belong the blue-green algae which do not concern us in human parasitology.

In the phylum Thallophyta we have the algae, fungi and lichens. Only the fungi are of medical importance.

In thallophytes there is no distinct differentiation into root, stem and leaf. The plant body may consist of one cell or many cells forming the thallus.

Nomenclature.—The name of a plant should be a binomial and formed by the generic and specific name as for animals. It is generally held that the name of a plant genus, while valid only for one plant genus, may also be used for an animal genus, but it is considered advisable in naming a new plant genus to avoid a name belonging to an animal genus.

The terminations for plant orders, families, etc., differ from those in zoological nomenclature, the accepted ones being *ales* for orders, *aceae* for families, *oideæ* for subfamilies, *eae* for tribes and *inae* for sub-tribes.

Fungi

The division Fungi of the phylum Thallophyta embraces the following classes of interest medically: Phycomycetes, Ascomycetes and Hyphomycetes or Fungi imperfecti.

Fungi do not have cholorophyll. In their simplest form they are ramifying filaments called hyphae. A network made up of vegetative hyphae intertwined in tangled threads is termed the mycelium. Growth may be either by addition of new hyphae (apical growth) or by division in a single hypha (intercalary growth). The hypha may be a single cell or many cells separated by septa.

Certain types of fungi have hyphae which form fruiting branches called conidiophores which support specialized cells called conidia. These conidia are asexual in origin. In some fungi resisting structures are formed which withstand unfavorable environment. These are named chlamydospores. Other groups form hard bodies called sclerotia which may be distinctive of species. They may resemble sexual fruiting bodies. While sexual bodies offer a satisfactory method of differentiation, they are not present with all fungi. Diseases caused by fungi are known as mycoses.

Phycomycetes

These produce a copious network-like mycelium, which is nonseptate, and reproduce asexually by means of a sporangium, a case-like structure borne on the clubbed extremity of an erect hypha (columella) and containing numerous spores or, as in the case of the suborder Oomycetes, reproduction is by heterogamy. (Dissimilar sexual cells—a smaller male, antheridium, and a larger female, oogonium. By fertilization by antherozoids from the antheridium penetrating the oösphere we have oöspores.)

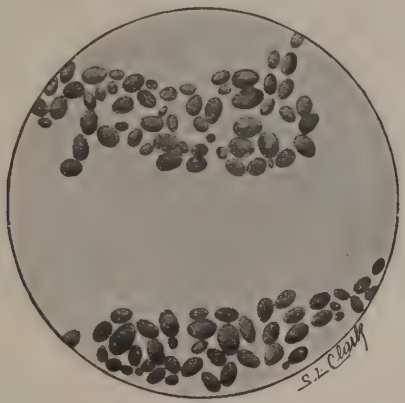


FIG. 49.—Yeast cells. *Saccharomyces cerevisiae.* (*Coplin.*)

The suborder Zygomycetes reproduces either asexually (a sporangium filled with spores) or by isogamy (two similar but sexually differentiated cells conjugate and form on fusion a zygospore.)

Belonging to this suborder we have four families, only one of which, the Mucoraceae, is of importance medically. In this family we have three genera: *Mucor*, without rhizoids; *Rhizopus*, with rhizoids and unbranched aërial hyphae, and *Rhizomucor*, with rhizoids and ramified mycelium.

Under anaerobic conditions the nonseptate mycelium of these fungi may break up into short septa resembling yeasts.

Our knowledge of parasitic moulds of the class Phycomycetes is in an uncertain state. The pathologic processes which they are supposed to produce are ill-defined, the cases are few, and while some of these moulds are pathogenic for animals their importance in human pathology is not great.

Two species of *Mucor* are of slight pathogenic importance:

1. *Mucor mucedo* and 2. *Mucor corymbifer*. These molds develop especially in external cavities as nasopharynx and external ear.

Pulmonary and generalized infections have also been reported. The pathogenic species have smaller spores and grow best at 37°C. The thick, coarse, cotton-like mould seen in horse manure is a *Mucor*. The sporangium, the organ of fructification, contains the spores within its interior. The *M. mucedo* has thick silver-gray mycelium, with large sporangia, 150μ in diameter, containing oval spores, 5 × 9μ. The *M. corymbifer*, which has been reported from a generalized infection, considered as typhoid, shows a snow-white mycelium. The sporangia are 20 to 40μ and the spores about 3μ in diameter.

Rhizomucor parasiticus has been reported from the sputum of a woman with a condition resembling phthisis.

Rhizopus niger has a columella which becomes distorted into a mushroom shape after the spores have been discharged from the sporangium. This mould has been considered as the cause of a mycosis of the tongue.

Ascomycetes

In this class are included many of the parasitic moulds. The most distinctive characteristic is the formation of ascospores in an ascus (little sac).

It is an enlarged extremity of a hyphal branch in which a definite number of spores, usually eight, is formed. The ascus usually ruptures at its tip. Other members of the class are formed from hyphae by the separation of cells in succession from the free cells. The mycelium is septate.

The class is divided into those with naked asci (Gymnoascales) and those having a perithecium or investing layer about the ascus or asci (Sphaeriales).

Gymnoascales.—Belonging to the order Gymnoascales we have 1. the family of Saccharomycetaceae, which reproduce by budding and in which the asci are without any semblance of a sheath, and 2. a family in which there is an indication of the formation of a perithecium—the Gymnoascaceae.

Saccharomycetaceae.—There are four genera: *Saccharomyces*, *Endomyces*, *Cryptococcus* and *Coccidioides*.

Saccharomyces.—These reproduce by budding, have ascopores and no mycelial-like threads.

S. cerevisiae.—This is the ordinary yeast fungus. Used at times as an antiseptic. It is also used in treatment of beriberi as it is very rich in vitamins (water-soluble B).

S. anginae.—Found in a case of angina.

S. blanchardi.—Found in a jelly-like tumor mass of the abdomen. The budding cells varied from 2 to 20μ. Probably identical with *S. tumefaciens*, reported as the cause of a subcutaneous tumor about region of Scarpa's triangle.

Endomyces.—Forms spores in the interior of filaments, or by ascus formation or by chlamydospores (resistant spore-like structures with a thick membrane which project from the extremities or sides of the hyphae as bud-like structures).

E. vuillemini.—One of the organisms of thrush. It produces a false membrane, especially on buccal surfaces, which is easily detached and beneath which the mucosa is intact. Grows only in acid media; hence propriety of alkaline treatment. Some authorities consider the genus *Endomyces* as the same as *Monilia* or *Oidium.*

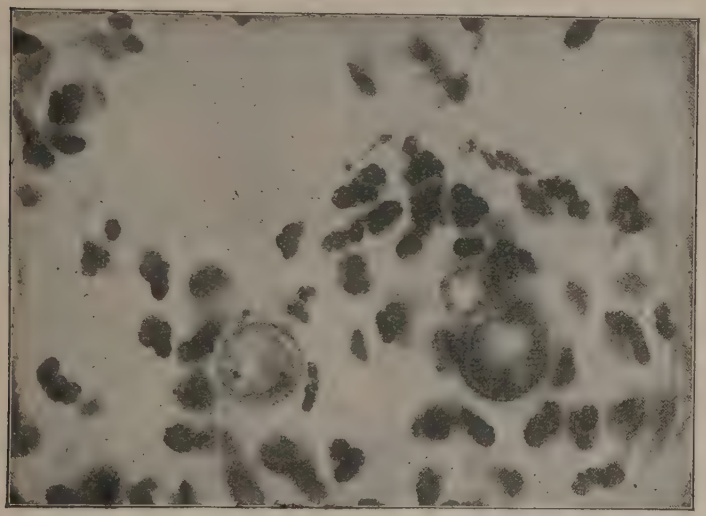


FIG. 50.—*C. gilchristi.* Doubly contoured organisms found in oidiomycosis (blastomycosis.) (*From Buschke after Hyde and Montgomery.*)

Cryptococcus.—Reproduces by budding, but ascospore formation not observed. Not a well recognized genus. The diseases caused by it are termed blastomycoses.

C. gilchristi.—The infection with this organism is usually termed blastomycosis or blastomycetic dermatitis. The causative organism, *Cryptococcus gilchristi,* is found in the purulent discharge as oval to round, doubly contoured, budding yeast-like cells (10 to 16μ). In cultures we have formation of a mycelium resembling that of an oidium. The lesions may be solely cutaneous or generalized, in which latter case the lungs are apt to be involved giving a condition resembling pulmonary tuberculosis.

C. linguae-pilosae.—This is a more or less elongated yeast-like organism and supposed to be the cause of black tongue, a benign affection of the lingual papillae.

Coccidioides immitis.—The infection with this organism is usually designated coccidioidal granuloma; it is very rare and fatal infection. The causative organism, *C. immitis,* is somewhat similar in cultures to *Cryptococcus gilchristi*

but differs from it in tissues in that it gives rise to endogenous spore formation in the cells found in the granulomatous material. The spores are about 3μ in diameter and contained in a large cell (30–60μ) which does not bud. We may have skin lesions accompanying visceral involvement or the latter alone. When involving the lung the infection closely resembles pulmonary tuberculosis. The spores metastasize readily by way of the lymphatics involved and we may have a picture of pyaemia. Skin lesions, when present, are ragged and punched-out. About 40 cases have been reported, chiefly from California.

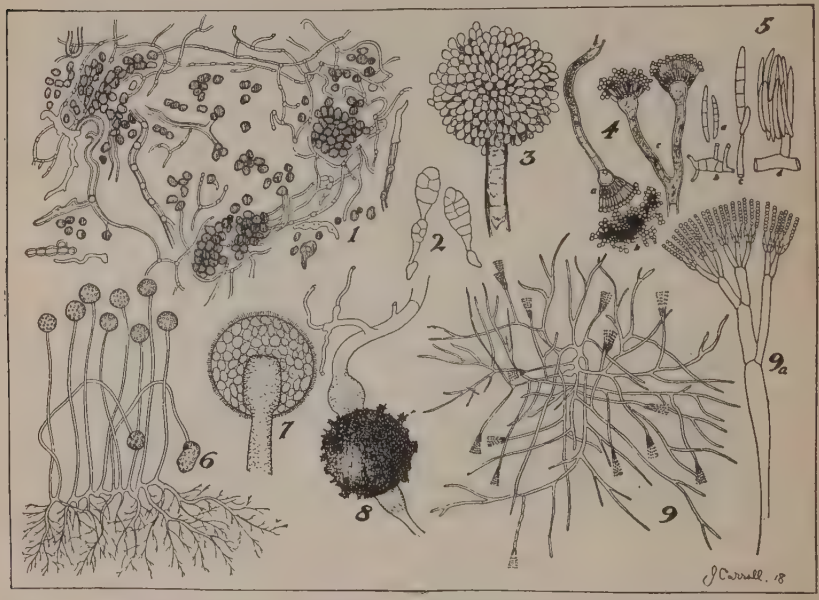


FIG. 51.—1, Tinea versicolor scrapings (*Malassezia furfur*). 2, *Alternaxia*. 3, and 4, *Aspergillus*. 5, *Fusarium*. 6, 7, 8, *Mucor*. 9, 9a, *Penicillium*.

Yeast-like bodies.—The small bodies, about 3μ, in the *Molluscum contagiosum* cells are thought by some to be yeasts. They are more probably artefacts in degenerated epithelial cells. Plimmer's bodies in cancer cells have been placed in this group, but they are probably not parasites.

Gymnoascaceae.—Belonging to this family we have the genera *Trichophyton*, *Microsporum*, *Achorion*, *Endodermophyton* and *Epidermophyton*.

Trichophyton.—The fungi of the genus *Trichophyton* cause the affections generally known as the large-spored ringworms. The spores are in chains and may be found inside the hair or both outside and inside. Many of them are parasites of

animals, especially the horse and the cat. The spores are from 5 to 15μ. Some fifteen species may infect man.

The mycelium is greatly segmented, shows simple or dichotomous branching, and produces spores within the mycelium.

T. tonsurans.—Give a crater-like culture with fine marginal rays. Fungus mostly inside the hair. Causes most of the large-spored scalp ringworms and many body cases. It is the *T. megalosporum endothrix* of Sabouraud. The short, diseased fragmented hairs are mouldy-looking. The spores are 5 to 6 microns in diameter.

T. sabouraudi.—Has a heaped-up festooned sort of culture. There is a similar fungus with a violet culture. These cause some of the scalp and beard ringworms. It is easily dissociated in a 2 or 3% solution of caustic potash while *T. tonsurans* is hard to break up. The hairs are broken off close to the skin, hence "black-dotted ringworm."

T. mentagrophytes.—This is the *T. megalosporon endoectothrix* of Sabouraud. The external spores are in chains or in short mycelial threads, not mosaics of spores, and are of very unequal size (3 to 15 microns). The internal spores are scarce and are from 5 to 6 microns in diameter. To examine pull out downy hairs from the periphery of the lesion rather than the dead central ones. There are varieties from horse, cat, and bird. The lesions are more inflammatory than those of the endothrix class. Most of the beard and body ringworms belong to this group—very few scalp cases. The lesions are often of a pustular type. The cultures are finely rayed. Some give yellow cultures, others white and one derived from birds a rose-colored culture.

Microsporum.—The fungi of the genus *Microsporum* are generally known as small-spored moulds. There are about a dozen species which may infect man. Microsporum infections tend to recover spontaneously in children over 15 years of age.

Microsporum audouini.—This organism is the cause of a very common and highly contagious affection of the scalp in children in England and France; less common in other countries.

It is almost never seen in the tropics. It almost exclusively affects the hairy scalp. The spores are 2 to 3μ in diameter. The broken stump of the hair is characteristic. The fungus is packed as a mosaic of spores, forming a white sheath, chiefly on the outside of the hairs. It gives a downy-white culture.

Achorion schoenleini is the cause of favus. The disease is characterized by the scutulum or favus cup, a sulphur-yellow pea-sized depression with a central lusterless hair. Affected hairs may not, however, show a cup. Favus is not so contagious as ringworm. It chiefly affects the hairy scalp, but may invade also the nails and even the body.

Microscopical examination shows great irregularity of spores and mycelium, the latter being irregularly disposed and of varying thickness and length, and wavy instead of straight as in *Trichophyton*. There is also the greatest irregularity in the refractile favus spores—they are gnarled and bizarre-shaped, in contrast to the regular ovals or spheres of the ringworm fungus. Cultures show ridges or convolutions. Species of *Achorion* occur on birds and animals, and the human species may infect certain animals.

Endodermophyton concentricum.—Castellani considers this rather than *Aspergillus concentricus* as the causative fungus of tinea imbricata.

It was formerly supposed that the causative fungus was *Aspergillus concentricus* but Castellani has demonstrated that fungi of this genus, when present, are merely accidental. He has isolated in cultures what he considers the causative fungus,

Low Power High Power

FIG. 52.—*Epidermophyton cruris*, from skin scrapings. (Low and high power.)

Endodermophyton concentricum. He treated scales for ten minutes with absolute alcohol and then placed single scales in a series of tubes of maltose bouillon. The fungus grows between the rete malpighii and the external epidermal layers forming a network of mycelial threads about 3 microns broad.

Another fungus cultured from tinea imbricata scales is *Endodermophyton indicum.* Inoculation of this organism in pure culture produced the disease.

The characteristics of the genus *Endodermophyton* are the growth of a mycelial network between the rete malpighii and the superficial epidermal layers, and in cultures the occurrence of mycelial filaments alone, there being no conidia-bearing hyphae.

Epidermophyton cruris. See *Microsporoides minutissimus*, page 207.

This fungus causes "dhobie itch," an affection probably better known to Europeans than any other tropical skin disease.

This name dhobie or washerman's itch has been given on account of attributing the disease to infection of the underclothing while being washed in the pools or streams along with the garments of those who have this skin disease. This, like every other widespread view, has probably some foundation but cannot be verified. It is the eczema marginatum of Hebra. This affection is caused by various species of *Epidermophyton*. The genus differs from *Trichophyton* in that it never invades the hair or hair follicles.

The species which have been frequently reported are *Epidermophyton cruris*, *E. perneti* and *E. rubrum*. The mycelium is about 4 microns broad and the spores about 5 to 6 microns. All of these fungi can be cultured on Sabouraud's maltose agar, growth appearing in about a week, except in the case of *E. perneti*, which grows more rapidly.

Species of the genus *Epidermophyton* are remarkably common in temperate and tropical regions and cause much annoyance to soldiers, sailors and civilians. Eczematoid affections of hands and feet are caused by them. These are generally diagnosed pompholyx or eczema and treated accordingly with failure to cure. Microscopically they are easy of diagnosis and persistence in treatment (salicylic ointment) will cure them.

SPHAERIALES.—In this order we have to consider the family Perisporiaceae.

Perisporiaceae.—In this family the asci are completely inclosed by the investing membrane, the perithecium. When this rots the spores are set free. There are three genera of interest, *Penicillium, Aspergillus* and *Sterigmatocytis*.

In *Penicillium* we have vertical branches with strings of conidia. In *Aspergillus* these conidia arise from a globular termination of the hypha.

Penicillium.—While *Penicillium* does at times form perithecia, yet it characteristically shows chains of spores. The common *P. glaucum* resembles a hand with terminal beads, more than the hair pencil, from which the name is derived.

P. crustaceum.—Is the common blue-green mould. It has been deemed pathogenic in cases of chronic catarrh of the Eustachian tube and in gastric hyperacidity.

P. montoyai.—Cause of violet pinta.

Aspergillus.—These have sterigmata carrying chains of spores, these sterigmata being little processes projecting out from the knob-like termination of the aërial hypha (columella). Of the pathogenic Aspergilli we have:

1. *A. fumigatus*.—This has been considered as the cause of pellagra. A pulmonary mycosis resembling phthisis may be due to this species.
2. *A. repens*.—This has been found in the auditory canal and may produce a false membrane.
3. *A. flavus*.—This has been found in the discharges of chronic ear diseases.
4. *A. concentricus*.—Formerly considered as the cause of an important tropical ringworm, tinea imbricata. In this disease, scales are dry, like pieces of tissue-paper. There are generally about four rings which do not heal in the center.

General appearance is that of watered silk. There are no inflammatory lesions. Common in Malay peninsula. Also found in some parts of the Philippines and in China. Some authorities consider the fungus to be a *Trichophyton*, others an *Endodermophyton*.

5. *A. pictor*.—This is the cause of a skin affection of Central America known as pinta, caraate or mal de los pintos. The disease is attended with a mangy odor. In the affection, colored spots appear on the skin, chiefly on face, forearms, and chest. Spots are of various colors.

At first it was thought that the different colors shown by the eruption were due to varying depths of the proliferating fungi in the skin layers but it is now known that they are specific with the several infecting species.

The pure violet pinta is caused by *Aspergillus pictor*, while the grayish-violet one is due to *Penicillium montoyai*. A species of *Monilia* causes the white variety and two species of *Montoyella* a black and a red variety respectively. The genus *Montoyella* is stated by Castellani to have both slender and thick mycelial threads, from the thicker of which spring delicate hyphae terminating in pear-shaped conidia.

Material scraped from the lesions and mounted in liquor potassae shows the fructification terminations characteristic of *Aspergillus* or *Penicillium* in the violet or gray-violet varieties while the white, black and red ones show only mycelial threads and scattered spores. These pinta species of fungi can be cultivated on Sabouraud's medium.

Montoya thinks that the pinta fungi lead a saprophytic existence in the waters of mines or other places with a constant high temperature, and states that he has obtained pure cultures from such sources.

Sterigmatocytis.—This genus has chains of conidia, similar to those of *Penicillium*, but borne on other short chains, which arise from the clubbed aërial hyphae (conidiophores). These are called secondary and primary sterigmata, respectively.

S. nidulans.—This fungus has been found in cases of otomycosis and in the white granules of mycetoma.

Hyphomycetes

In this class are grouped certain genera which cannot properly be assigned to any of the other classes. They are also designated Fungi imperfecti, for the reason that the fruiting bodies characteristic of the other classes have not been observed satisfactorily. Chalmers and Archibald recognize *mycetoma* as a condition caused by the invasion of fungi, forming grains composed of hyphae, and at times chlamydospores, embedded in a matrix. Such lesions usually show eosinophile bodies. With *paramycetomas* the fungus growth does not appear as grains and the condition shows eosinophile bodies. *Pseudomycetomas*

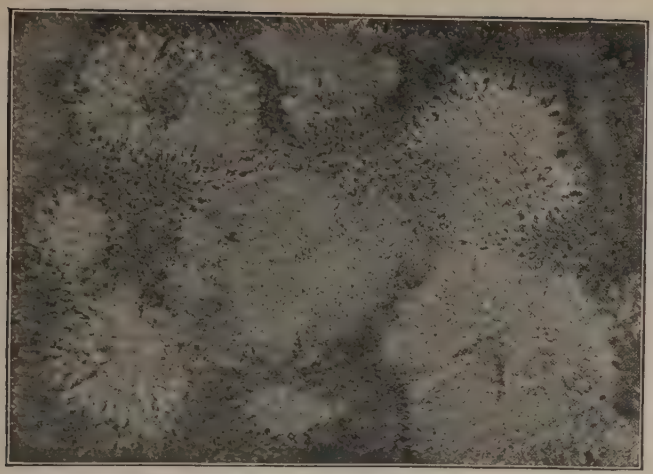


FIG. 53.—Actinomyces granule crushed beneath a cover glass, showing radial striations in the hyaline masses. Preparation not stained; low magnifying power. (*McFarland after Wright and Brown.*)

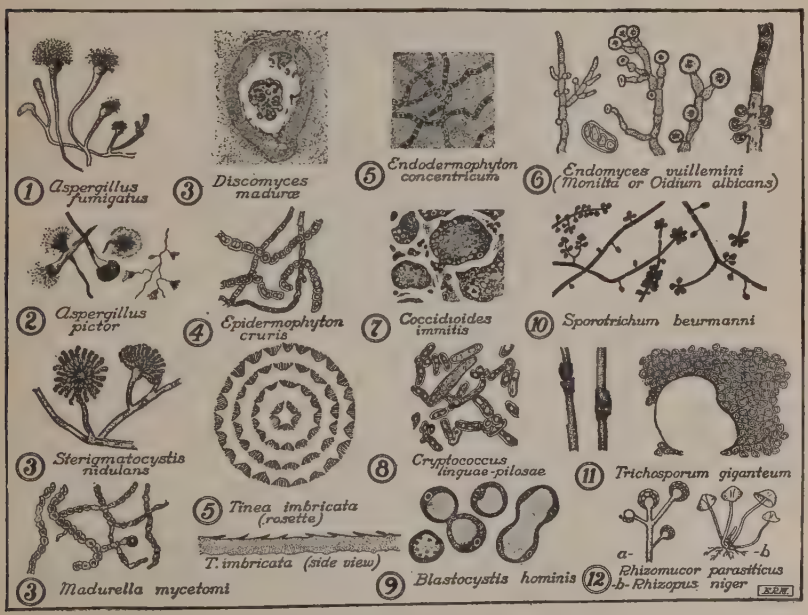


FIG. 54.—Important tropical fungi.

fail to show either fungal growth or eosinophile bodies, and many have the histology of atypical epitheliomata.

The *mycetomas* are divided into *maduromycoses*, with grains showing large segmented mycelial filaments, possessing well defined walls and usually chlamydospores, and the *actinomycoses* with very fine, nonsegmented mycelial filaments, with ill-defined walls and no chlamydospores.

The maduromycoses are divided into the black, white or yellow and red ones, according to the color of the grains.

Discomyces bovis.—This is the well known ray fungus, the cause of actinomycosis. In man it is at times found in chronic suppurative conditions attended with much granulation tissue. Such pus may show small yellow-gray granules about the size of a pin's head. When spread out between two slides the central portion shows a network of mycelium with bulbous thread-like rays going to the periphery. The "clubs" at the periphery are degenerate structures and do not stain by Gram. The central mycelium is Gram-positive. This mould is essentially an anaerobe and should be cultivated in a deep glucose agar stab. It may also be cultivated in bouillon. In this it grows at bottom. Growth is dry and chalky. In diagnosis look for the little granules. Curetting of the sinuses may give the "ray fungus" when they are not found free in the pus.

Discomyces madurae.—This is a ray fungus found in the yellow "fish-roe" granules of madura foot. The disease is caused by the penetration of certain species of fungi into the tissues of the foot, although rarely the hand or some other part of the body may be affected. These species of fungus develop in granulomatous areas from which sinuses lead to the surface of the foot, in the discharges from which are found small granules resembling those found in the discharges from actinomycosis lesions.

As a rule only one kind of fungus is found in a case. The most common infection is that due to *Discomyces madurae* (*Nocardia madurae*) which is the fungus of the fish-roe-like granules of the pale or white variety of mycetoma. These, like the fungus of actinomycosis, *Discomyces bovis*, show a felted mycelium in the center and peripheral club-like structures. The granules are yellowish-white and vary in size from a pin's head to a small pea. The mycelial threads are very narrow, 1 to 1.5 microns. It grows aerobically and the cultures show slender mycelial threads which are Gram-positive. This is the organism of Carter's white mycetoma.

Some of the species of the pale, white or ochroid group of mycetoma fungi are *Indiella mansoni* (Brumpt's white mycetoma), *Nocardia asteroides* (Musgrave and Clegg's white mycetoma) and *Sterigmatocystis nidulans* (Nicolle's white mycetoma).

The cases caused by the black varieties are more rare and are characterized by the presence in the discharge from the sinuses of black gunpowder-like grains.

These hard, brittle, irregular grains are caused by various species of fungi of which the best known is Carter's black mycetoma (*Madurella mycetomi*). This species was cultured by Wright and first shows a grayish growth, later becoming black. Other black varieties of mycetoma are due to various other fungi. Bouffard's black variety is caused by *Aspergillus bouffardi*. De Beurmann's black mycetoma has as cause *Sporotrichum beurmanni*. Boyd and Shear have described a mycetomatous condition of the foot from Texas due to *Allescheria boydii*.

Besides the white and black varieties we also have a red variety of mycetoma. The fungus grains are quite small and reddish in color. It is not an uncommon infection in certain parts of Africa, as Senegal. The cause is *Nocardia pelletieri*. *Discomyces corougeaui* has been reported as the cause of juxta-articular nodules; but Breinl has been unable to verify the finding.

FIG. 55.—*Endomyces vuillemini*. Mycelial thread with four ripe chlamydospores; and conidia in the middle of the picture. (*After Plaut.*)

Malassezia furfur.—This is the fungus of tinea versicolor. It is common both in temperate and in tropical climates. It is characterized by dirty yellow spots about covered parts of the body. Scrapings show a profusion of mycelial threads and interspersed spores. It is very difficult to cultivate. The organism usually termed the "bottle bacillus" is really a fungus having the characteristics of the genus *Malassezia*. It is thought to be the cause of pityriasis of the scalp.

FIG. 56.—Thrush fungus. (*Kolle and Wassermann.*)

Microsporoides minutissimus.—It is characterized by its narrow mycelium and small spores. This is generally considered as the cause of erythrasma. It is one type of dhobie itch, a very common intertrigo of the tropics. Various other fungi also are found in this affection. Castellani considers the chief cause of dhobie itch to be *Epidermophyton cruris*. (See p. 202.)

Clinically this affection shows festooned areas of a bright red color which tend to clear up in the center, becoming fawn color. As a result of the intolerable itching and scratching the affection tends to spread from its favorite sites—the inner sur-

faces of the thighs and the scrotum. The spores and mycelium are abundant at the onset but, later, one may not find any evidence of the mould. In some of the rapidly spreading cases I have found a symbiosis of fungus and coccus, the bacterial elements lying packed in aggregations scattered through the mycelial ground work. Culturally these cocci were *S. aureus*.

Monilia albicans (*Oidium albicans*).—Castellani separates *Monilia* from *Endomyces* in that it does not show the asci and internal spores of the latter. In cultures it gives budding yeast-like growths and mycelial threads. On Sabouraud's medium it gives a whitish growth. It slowly liquefies gelatin and blood serum and, after acidifying, clots milk. It is recognized as the organism of thrush. Bahr found this fungus in the deep layers of the tongue as well as in the oesophageal and intestinal coatings of sprue cases.

Ashford is convinced that a species of *Monilia*, which he is sure is distinct from *M. albicans*, is the cause of sprue. He states that this fungus is common in the bread of Porto Rico. He has recovered the organism from sprue lesions and has produced monilia septicaemia in rabbits inoculated with the sprue fungus. This fungus is quite pathogenic when first isolated from sprue lesions.

In internal organs the mycotic areas are not associated with pus formation. On two occasions he has produced stomatitis in animals by feeding experiments.

Monilia tropicalis.—Castellani has reported this fungus as the cause of a bronchomycosis. It does not coagulate milk or liquefy gelatin. The growth on Sabouraud's medium is mainly of yeast-like cells.

M. candida.—This fungus was found in white patches on tongue of a child. The conidia are 7 to 8 microns and the mycelium 1 to 1.5 microns in diameter.

Boggs has recently isolated a *Monilia* which he considers as closely related to *M. candida*. The patient was at first thought to have a mammary carcinoma with axillary gland metastases. Later on there was a severe cough with abundant reddish-gray sputum which showed mycelium and yeast-like cells. Cultures on glucose agar, potato, etc., at 37°C. and at room temperature, gave a moist glistening whitish growth which, when examined, showed only yeast-like cells, no mycelial growth. Hyphae showed only in later cultures. There was a fair growth in milk with, after three or four weeks, an aklaline reaction and firm coagulum. Slight acid production in glucose but none in lactose, saccharose or mannite. Mycelial growth was more rapid in anaerobic cultures than in aerobic ones. Boggs notes that his *Monilia* is morphologically indistinguishable from the *Monilia* of sprue. The buccal mucosa of this case did not show any abnormalities.

Trichosporum giganteum.—This is the cause of a disease of the hairs known in Colombia as "Piedra," so called from the small gritty masses along the length of the hair. These spores are arranged like mosaics about the hair.

Sporotrichum schencki.—This fungus has a narrow mycelium (2μ) and branches in all directions. The spores appear as little grape-like clusters of oval spores (3 to 5μ) at the end of a filament. It is readily cultivated, showing as a small white growth about the second day. Another species is *S. beurmanni.*

The fungus of sporotrichosis develops in tissue by budding, not showing the mycelial growth seen in artificial cultures. Potato makes a good medium, on which the cultures often show pigmentation.

This mould produces indolent, glistening, subcutaneous tumors which are painless. They may ulcerate and give off a brownish discharge. They resemble tuberculous or syphilitic lesions. The parasites assume a bacillary form in the discharges, but are rarely found in them, so that for diagnosis one should make cultures on agar plates.

Nocardia.—Certain organisms which resemble both moulds and bacteria, having branching filamentous forms and at the same time having a spore-like method of reproduction, are known under the names *Streptothrix* or better *Nocardia.* It is chiefly in various pathological processes of the lungs that they have been observed, but in addition they have been noted in lesions of the brain, glands, kidney and subcutaneous tissue. These infections are most likely to be confused with phthisis and glanders. The organisms are easily cultivated and in staining reactions are midway between tubercle bacilli and actinomycosis.

Castellani uses the generic name *Nocardia* for *Discomyces.*

FIG. 57.—*Sporotrichum schencki.* Cultures on the glucose peptone agar of Sabouraud. (*After Gougerot.*)

METHODS OF EXAMINATION

The most expeditious way to examine for fungi is to treat the scales or hairs with a 10% solution of caustic potash or soda. Then crush between two slides; heat moderately over the flame and examine.

Tribondeau's method is to treat the scales with ether, then with alcohol, and finally with water. Next put the sediment (it is convenient to use a centrifuge) in a drop of caustic soda solution. Cover with a cover glass, and after the preparation has stood about an hour run glycerin under the cover glass.

A very satisfactory method is to scrape the scales with a small scalpel, and smear out the material so obtained in a loopful of white of egg or blood serum on a glass slide. By scraping vigorously the serum may be obtained from the patient. After the smear has dried, treat it with alcohol and ether to get rid of the fat. It may then

be stained with Wright's stain or by Gram's method. The ordinary Gram method may be used, or the decolorizing may be done with aniline oil, observing the decolorization under the low power of the microscope.

Yeasts are best examined in hanging drop on a plain slide with vaselined ring, as given under "Blood."

An excellent way to examine moulds is to seize some of the projecting sporangia from the surface of a plate with forceps and mount in liquid petrolatum. I have found that moulds in scales from skin or from infections of various mites or insects will show a growth in this medium when mounted on a slide and covered with a cover glass.

For the microscopic study of moulds it is well to clear and mount them in lactophenol (carbolic acid, 20 cc.; lactic acid, 20 cc.; glycerin, 40 cc.; water, 20 cc.). This may be tinted with some dye.

Cultivation of Fungi

Moulds grow well on media with an acid reaction, so that by adjusting the reaction to +2, or even higher, we permit the growth of the fungi, but inhibit bacterial development.

Glycerin agar, bread paste, or potato media are all suitable, but the best media are those of Sabouraud:

Conservation medium (for preserving stock cultures).

Peptone	30.0 Gms.
Agar (shred)	15.0 Gms.
Tap water	1000.0 Gms.

Differentiation media (two of these are used—a maltose medium and a glucose medium).

Maltose	40.0 Gms.
Peptone	10.0 Gms.
Agar (shred)	15.0 Gms.
Tap water	1000.0 Gms.

The glucose medium is made by substituting glucose for maltose in the above formula.

In each case the ingredients are added to the water and all placed in a cold autoclave and the pressure allowed to rise in both outer and inner jacket simultaneously until it has reached 15 pounds. The autoclave is then shut off and allowed to cool down slowly. When autoclave has cooled, the media is filtered through cotton, tubed, and then sterilized the same as above. Upon removal from the autoclave, the tubes are slanted and allowed to cool in this slanting position.

The media is not titrated or the hydrogen-ion concentration adjusted in any way, Sabouraud claiming that the addition of either acid or alkali spoils the media.

Before inoculating media with moulds, some recommend placing the material in 60% alcohol for one or two hours to kill the bacteria. The moulds withstand such treatment.

In cultivating moulds, small Erlenmeyer flasks, containing about ¼ in. of media on the bottom, will be found suitable for the development of the colonies. In order to isolate the mould we may take the hair or scales on a sterile slide and cut them into small fragments with a sterile knife. Then moisten a platinum loop from the surface of an agar slant, touch a fragment with the loop, and when it adheres transfer it to the agar slant. Make four or five inoculations on the surface, and from suitable growth, after four to seven days, inoculate the medium in the Erlenmeyer flask. Esmarch roll cultures are better than flask ones.

Plaut recommends: Place the mould material between two sterile glass slides. Seal one edge with wax and place the preparation in a moist chamber for four to seven days. From the fungus growth developing inoculate the medium in the Erlenmeyer flask. A Petri dish containing several layers of thoroughly moistened filter paper in top and bottom makes a satisfactory moist chamber.

Hanging block cultures.—One of the best methods of identification of moulds is to fill the concavity of a hollow slide, which has been flamed for sterilization, with melted Sabouraud agar or other media suitable for moulds. The surface is then inoculated with material from the colony to be studied and a flamed (sterile) cover glass applied. In a similar method, which was devised for the study of bacterial cultures, Hill used 1 cm. squares of agar, cut out from sheets made by pouring melted agar into Petri dishes. Another method is to let a large drop of melted agar spread over a sterile cover glass and then inoculate the film of medium and adjust over a concave slide. These methods are excellent for bringing out the mode of fructification of moulds which can then be studied satisfactorily with high powers.

For the study of the morphology of *Monilia* in cultures, Boggs used stab cultures in 15% gelatin. The growth in the tube was hardened in 10% formalin, the glass cracked off and sections of the gelatin column cut across at any desired level. These blocks were sectioned with the freezing microtome; stained in dilute aqueous fuchsin (1 to 30) for several hours; then differentiated in saturated solution of citric acid until nearly decolorized. The sections were floated on slides, air-dried without blotting, cleared in xyol and mounted in balsam.

For staining fungi in sections of tissue Busse recommends the following method: 1. Haematoxylin, 10 to 15 minutes; then wash in tap water. 2. Carbol fuchsin (1 to 20), 30 minutes or over night. Decolorize in alcohol for a few minutes, then pass through absolute alcohol and xylol to mount in balsam. The moulds are red.

Specific name	Synonyms	Lesions produced	Diagnosis	Remarks
Mucor corymbifer	Lichtheimia corymbifera.	Mycosis of (1) ear; (2) nose; (3) lungs; (4) generalized.	1. Microscopic. 2. Culture.	Pathogenic for animals (rabbits).
Mucor mucedo	Mucor vulgaris.	2 cases of pulmonary infection; 1 case bronchopulmonary mycosis.	1. Microscopic. 2. Culture.	Causes May's disease in bees.
Rhizomucor parasiticus		Found once in patient supposed to have tuberculosis; recovered under K.I.	Mycelial threads and spores in sputum.	Pathogenic for guinea pigs and rabbits.
Rhizopus niger	Mucor niger.	One case of black mycosis of tongue.	Rhizoids and umbrella-shaped sacs (when spore-free) in culture.	Not pathogenic for rabbits.
Saccharomyces cerevisiae		None.	Budding growth in preparations from cultures.	Produces alcohol; rich in water-soluble B vitamin.
Saccharomyces anginae		One case of angina.	Four-spored asci on gelatin, ferments saccharose.	Pathogenic for mice, guinea pigs and rabbits.
Saccharomyces blanchardi		One case of gelatinous peritonitis.	Mucoid growth on potato, whitish and darkening.	Generalized fatal mycosis in rabbits.
Endomyces vuillemini	E. albicans; Monilia albicans; Oidium albicans; Saccharomyces albicans.	Cause of thrush.	Microscopic and cultural.	Produces a generalized mycosis in rabbits. Agglutinins, antitoxins and mycolysins produced (Brumpt).
Cryptococcus gilchristi	C. hominis (?); Mycoderma dermatitis; Blastomyces dermatitis.	Blastomycetic dermatitis.	Budding, double contoured yeasts in microscopic preparations—is cultivable.	The dog has been successfully inoculated.
Coccidioides immitis	C. pyogenes. Oidium {coccidioides; protozoides; immitis; pyogenes.	Dermatitis with miliary abscesses. Infection often is generalized in organs causing death.	Round bodies with spores from tissues. Grows as a hyphal mould on media.	Monkey, rabbit, guinea pig and mouse capable of infection.

Trichophyton sabouraudi.....	T. acuminatum.	"Black-dot ringworm" of scalp; tinea circinata; onychomycosis; and dry trichophytosis of the beard.	NaOH and slight pressure breaks mycelium into spores 5–7μ in diameter.	Endothrix ringworm usually of human origin, is difficult to recognize and is intractable to treatment.
Trichophyton tonsurans.....	T. megalosporum endothrix; T. crateriforme; Trichomyces tonsurans.	Tinea capitis and corporis and onychomycosis.	Large and resistant mycelium.	Grows well with a velvety colony at first, finally powdery.
Trichophyton violaceum.....	.....	Tinea barbae; ringworm of scalp with bald patches.	Moist brownish culture, later becoming violet.	Has been recovered from ringworm of the scalp and of the beard.
Trichophyton mentagrophytes.....	T. gypseum; Microsporum mentagrophytes; Sporotrichum mentagrophytes; Trichophyton asteroides.	Kerion Celsi (scalp and beard), on glabrous skin an agminated folliculitis.	Produces colony heaped in center with rayed periphery.	A horse ringworm, also pathogenic for guinea pigs.
Epidermophyton cruris.....	Trichophyton cruris; T. inguinalis; Epidermophyton inguinale; T. castellanii.	Eczema marginatum (Hebra); Dhobie itch; eczematoid ringworm of the extremities resembling pompholyx.	Clear epidermis from new vesicle in 10 per cent. NaOH for microscopic examination. Culture on maltose agar	A cosmopolitan mycosis; chief cause of foot infections of soldiers and sailors.
Endodermophyton concentricum...	Lepi dophyton concentricum; Aspergillus lepidophyton; Aspergillus tokelau; Endodermophyton tropicale.	Tinea imbricata (Tokelau).	By NaOH preparations of scales and by culture of the mould.	A widespread mycosis of the Eastern tropical belt.
Montoyella nigra.....	.....	Produces black pinta.	Microscopical and by culture.	One of the several mycotic organisms of pinta.
Montoyella bodini.....	.....	Found in red pinta.	Microscopical and by culture.	One of the several mycotic organisms of pinta.
Microsporum audouini.....	Trichophyton decalvans; Trichomyces decalvans; Sporotrichum audouini; Trichophyton microsporon.	Ringworm of scalp, rarely of skin in children.	Microscopical and by culture.	M. audouini does not infect animals. Some 15 species of this genus (many of animal origin) will infect children. Causes 90 per cent. of child ringworm in England and France.
Achorion schoenleini.....	Oidium schoenleini; Oöspora porriginis.	Various forms of favus of scalp, nails and gastrointestinal tract (rare).	By presence of scutulum; NaOH mount; culture	Four species of Achorion are infective for man and animals. A. schoenleini will infect dogs.

Specific name	Synonyms	Lesions produced	Diagnosis	Remarks
Penicillium montoyai	P. pictor.	Found in pinta (caraate) of the grayish-violet type.	Penicillium-like fructifications may be seen in NaOH mounts; by culture.	Inoculable for man, doubtful for animals.
Penicillium crustaceum	Mucor crustaceum album; P. glaucum; P. expansum; Monilia digitata.	Found a few times in otitis media.	By NaOH mounts; by culture.	Intravenously it is pathogenic for rabbits, dogs and lambs.
Sterigmatocystis nidulans	Aspergillus nidulans.	Otomycosis; pharyngomycosis; mycetoma with white granules.	Microscopical of granules; culture.	Large intravenous injections pathogenic for rabbits.
Allescheria boydii		Mycetoma with white granules; one case from Texas.	Granules show spatulate bodies (conidiospores). Grows on solid media with the eventual production of black perithecia.	Animal inoculation unsuccessful.
Aspergillus fumigatus		Aspergillosis of various organs. Pulmonary, bronchial, aural and corneal have been described.	Culture the excretions.	Effects are mechanical and toxic. Laboratory animals susceptible.
Aspergillus pictor	Trichophyton pictor.	Pure violet pinta.	Identified by microscopic and cultural procedures.	
Aspergillus niger	Sterigmatocystis antacustica; Monilia pulla; A. nigricans.	Lesions of ear and lungs have been attributed to it.	By microscopic and cultural means.	A common saprophytic mould.
Discomyces bovis	Actinomyces bovis; A. bovis sulphurens; Bacterium actinocladothrix; Nocardia actinomyces; N. bovis; Streptothrix actinomyces; Cladothrix actinomyces.	Actinomycosis in cattle and man and mycetoma in man.	Microscopical examination of granules and culture.	Animal inoculations seem to have been unsuccessful:
Discomyces madurae	Streptothrix madurae; Nocardia madurae.	Produces mycetoma with the white grains of Vincent.	The grains are typical and the organism grows reddish on potato, optimum 37°C.	Animal inoculation has been unsuccessful.

Madurella mycetomi	Streptothrix mycetomi.	Causes mycetoma (Carter's with black grains); wide distribution.	Difficult to cultivate. A variety of the mycelial elements is noted in the black granules.	Animal inoculation negative.
Malassezia furfur	Microsporum furfur; Sporotrichum furfur; Oidium { furfur / subtile.	Pityriasis versicolor, a cosmopolitan mycosis.	Color of lesion, NaOH-microscopical preparation; culture is difficult; pinhead-sized colonies of clear yellow color develop on glycerin agar.	Rabbit may be experimentally infected.
Nocardia asteroides	Discomyces asteroides; Streptothrix eppingeri; Oöspora asteroides; Discomyces freeri.	Mycetoma; brain abscesses; peritonitis.	Mycelial units easily separated, then look like bacilli. Acid-fast. Like B. tuberculosis but grow on ordinary media.	Rabbit and guinea pig give lesions like those of tuberculosis.
Microsporoides minutissimus	Microsporon gracile; Sporotrichum minutissimum; Nocardia minutissima.	Erythrasma; some cases like dhobie itch.	Mycelium 0.6–1.3 μ in diameter. Easily fragmented, occasionally branched wine-red culture on potato (difficult).	Animal inoculations negative.
Monilia albicans	Monilia enterica; Monilia psilosis; Monilia intestinalis; Oidium albicans; Endomyces vuillemini (according to Brumpt); Parasaccharomyces ashfordi.	Supposed to be connected with etiology of sprue. Growth in sprue probably incidental to change in culture conditions throughout the gastro-intestinal tract.	Grows well on acid Sabouraud's media. From lesions shows budding forms (asci absent) or simple and ramified threads.	Produces general infection in intravenous injection in rabbits. Specific antibodies may be produced.
Monilia candida	M. bonordini.	Thrush-like lesions on tongue and buccal mucous membrane.	Yeast-like conidial elements. Resembles monilias found in sprue.	
Trichosporum giganteum		Piedra of Colombia and other South American countries.	Gritty nodosities on hairs show spores. Organism cultivable.	
Sporotrichum schencki	S. beurmanni; Sporothrix schencki.	Gummatous lymphangitis.	Yeast-like bodies sparse in lesions; easily cultivable; conidia occur in groups terminal or surrounding a filament.	Dog, cat, guinea pig, rats and mice are susceptible. In rats a generalized infection with orchitis is produced.

The diagnosis of the mycoses is practically always a matter for the laboratory. The main dependence is upon the microscopic method backed up by culture. Nearly every one of the parasitic moulds will grow aerobically, though most are slow growers. Most of them prefer temperatures of from 22° to 30°C. though some of the more confirmed parasites grow better at 37°. It is perhaps unnecessary to state that we should, in studying any supposed mycosis, remember the ubiquitousness of saprophytic species of fungi and that these may grow in exudates due to lesions resulting from the action of bacteria or protozoa.

The attempt to diagnose a mycosis by the demonstration of antibodies is a waste of time. Some of the moulds which produce deep-seated or generalized infections may produce anti-substances (agglutinins, precipitins, opsonins or mycolysins) but it is unlikely that those which grow in the upper layers of the skin (away from the superficial blood vessels) can do this. The chronicity of most of the mycoses would seem to lend support to the belief that antibodies are not produced. However, the ease of microscopic diagnosis makes the demonstration of antisubstances for this purpose unnecessary.

The subject of medical mycology is in a very unsatisfactory and unsettled condition. In the preceding table for much of which I am indebted to Brumpt there are given the specific names of the several recognized parasitic moulds, together with a brief statement of the lesions they are supposed to produce and the best method for diagnosis.

CHAPTER XI

BACTERIOLOGY OF WATER, AIR, MILK, ETC.

Bacteriological Examination of Water

General Considerations.—In a chemical examination as to the character of a water there are certain relations between the free and albuminoid ammonias, nitrates, nitrites, chlorides, etc., which indicate the probable animal as against vegetable origin of the organic matter present, yet the evidence they furnish is merely presumptive in nature; whereas, in a bacteriological examination of water the finding of organisms of the *coli-aerogenes* group may from a practical standpoint be considered as positive evidence of human faecal contamination. Theoretically, the possibility of organisms being present corresponding culturally to those of this group but derived from cereals is to be considered. Also the faeces of animals contain an organism which cannot be differentiated from the colon bacillus.

In detecting sewage contamination in water to which varying amounts of sewage had been added, it was found that the bacterial tests were from 10 to 100 times more delicate than the chemical ones.

As showing sewage contamination of water, the presence of organisms of the *coli-aerogenes* group is accepted as the most conclusive indication. The English authorities also consider sewage streptococci and the spore-bearing *B. enteritidis sporogenes* of value as indicators—the presence of sewage streptococci indicating very recent sewage contamination and that of the spore-bearer, in the absence of streptococci and colon bacilli, as evidence of sewage contamination at some period more or less remote.

In the United States the *coli-aerogenes* group is considered the indicator of sewage contamination, and all tests, presumptive or positive, are based on the presence of this group. However, the decision rests not solely upon finding orgainsms of this group but rather on their relative abundance. Thus the finding of one organism in 50 cc. of water would not have weight as showing contamination, but the presence rather constantly of these organisms in 1 cc. or less makes contamination of a water supply probable.

In collecting samples of water for bacteriological examination, the following points of technique should be observed:

1. The bottles, which should have a capacity of from 25 to 100 cc. should be sterile. Sterilization may be effected by steam in an autoclave at 15 lbs. (120°C.) for at least 15 minutes after the pressure reaches 15 lbs., or by rinsing with a little

sulphuric acid and subsequently washing out thoroughly with the suspected water before collection. The utmost care must be exercised that the fingers do not come in contact with the glass stopper or the neck of the bottle while filling it. If the specimen is to be sent some distance, it should be packed in ice to prevent multiplication of bacteria. Frankland states that when ice was not used a count of 1000 became 6000 in six hours, and 48,000 in forty-eight hours. In water packed in ice for a considerable time, however, the bacterial count may diminish. The time elapsing between collection and beginning the analysis should not be more than six hours for an impure water, and not more than twelve hours for relatively pure waters. During this interval, the temperature should be maintained as near 10°C. as possible. If there has occurred any departure from these standards of time and temperature, the fact should be noted in the report.

2. If collecting from city water supplies, secure the sample direct from the mains and let the water run from the tap a few minutes before collection. If the water be taken from a pond, stream, or cistern, be sure that the specimen comes from at least 10 inches below the surface. As sedimentation is the most important means whereby the self-purification of rivers and ponds is brought about, it will be understood that any stirring up of the mud on the bottom will enormously increase a bacterial count.

Technique of Bacteriological Examination.—1. The most practical method is to deliver the water from a graduated pipette into empty sterile petri dishes. The water should be deposited in the center of the plate and the melted gelatin or agar poured directly on the water and then, carefully tilting to and fro, mix the water and the media. One set of plates should be of gelatin and incubated at room temperature; a similar set should be of lactose litmus agar and incubated at 38°C. If the water is highly contaminated, it is necessary to dilute it; thus, with river water, which may contain from 2000 to 10,000 bacteria per cc., a dilution of 1 to 100 would be desirable. Dilution bottles should be prepared by filling them with such a volume of water that there will remain in them exactly 9 cc. or 99 cc., as desired, after proper sterilization in the autoclave. The volume required must be determined for the particular autoclave employed. A stock of sterile diluting water may be kept on hand and appropriate amounts added by means of sterile pipettes.

All sample bottles and dilution bottles must be shaken at least 25 times before removing water for the examination, and the water should be plated immediately after dilution.

Ordinarily it will be sufficient to deliver from a sterile graduated pipette 0.2, 0.3, and 0.5 cc. of the water in each of two sets of plates; one set for gelatin, the other for agar.

When gelatin is not at hand or convenient to work with, the gelatin plates may be replaced by others of lactose litmus agar for incubation at room temperature. After twenty-four hours at 38°C. or forty-eight hours at 20°C., the count should be made. A very convenient method is to pipette 1 cc. of the water into each plate and add to each 10 cc. of melted media. Counts should be made on plates showing more than 25 and less than 250 colonies.

For example, if forty colonies were counted on the gelatin plate containing 0.2 cc. of the water, the number of organisms would be 200 per cc. If ten colonies were counted on the agar plate containing 0.2 cc. and incubated at 38°C. the number of bacteria developing at body temperature would equal 50 per cc.

There is no strict standard as to the number of bacteria a water should contain per cc. Koch's standard of 100 colonies per cc. is generally given. It is by the qualitative rather than the quantitative analysis that one should judge a water.

If there should be very many colonies on a plate, it will be found that counting is facilitated if the surface be marked off into segments with a blue pencil. If colonies are very numerous, cut out of a piece of paper an aperture 1 cm. square. Count the number of colonies visible through this aperture when applied to different parts of the plate, and strike an average for each square observed. To find the number of

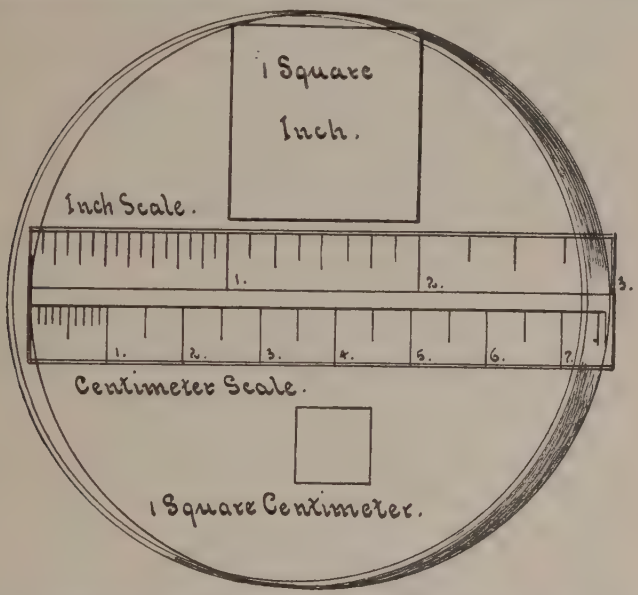


FIG. 58.—Estimating colonies on water plate. The diameter of the bottom of this Petri dish is 3 inches or 7.5 + centimeters. The area of a circle is equal to the square of the radius multiplied by π or $\frac{22}{7}$. $1\frac{1}{2}$ in. = radius. $1\frac{1}{2} \times 1\frac{1}{2} = 2.25$. $2.25 \times \frac{22}{7} = 7.07$ square inches. 3.75 cm. = radius. $3.75 \times 3.75 = 14.06$. $14.06 \times \frac{22}{7} = 44.1$ square centimeters. Number of bacterial colonies in 1 sq. in. averages, approximately, 75. Number in 7.07 sq. in. = 530. Number of bacterial colonies in 1 sq. cm. averages, approximately, 12. Number in 44.1 sq. cm. = 528.

such spaces contained in the plate, multiply the square of the radius of the plate in centimeters by 3.1416. Then multiply this number by the average per square centimeter, and we have the total number of colonies on the plate. This is the principle of the Jeffers disc.

The American Public Health Association recognizes the importance of the information obtained from a comparison of the number of organisms developing at 38°C. and those developing at 20°C. Bacteria whose normal habitat is the intestinal canal naturally develop well at body temperature, while bacteria normally occurring in water prefer the average temperature of the water in rivers and lakes. Consequently when the number of organisms developing at 38°C. at all approximates the

number developing at 20°C., there is a strong suspicion that sewage organisms may be present. Normal waters give proportions of 1 to 25 or 1 to 50, while in sewage-contaminated waters the proportion may be as 1 to 4 or 1 to 2.

In addition, the appearance of pink colonies on the lactose litmus agar is of great assistance in judging of a water. Both sewage streptococci and members of the *coli-aerogenes* group give pink colonies—those of the streptococci being smaller and more vermillion in color. Microscopic examination will differentiate the cocci from the bacilli. It is well to bear in mind that the pink colonies after twenty-four hours may turn blue in forty-eight hours from the development of ammonia and amines. Consequently the lactose litmus agar plates should be studied after twenty-four hours. A good water supply will rarely show a pink colony, while in a sewage-contaminated one the pink colonies will probably predominate.

In some countries the proportion of liquefying to nonliquefying colonies on gelatin plates is considered of importance. Certain sewage organisms belonging to the proteus and cloaca groups liquefy gelatin; consequently, if the proportion of liquefying to nonliquefying be greater than as 1 to 10, the water is to be considered under suspicion. The test is not regarded by American authorities as of any particular value.

2. Deliver into a series of Durham fermentation tubes containing lactose bouillon varying definite amounts of water to be examined. In tubes showing the presence of gas, the evidence is presumptive that organisms of the *coli-aerogenes* group are present. For the positive demonstration plates must be made from such tubes.

It is sufficient to deliver from graduated pipettes quantities of water varying in amount from 0.1 cc. to 10 cc. In our laboratory we inoculate with 0.1 cc., or 0.2 cc., 0.5 cc., 1 cc. and 10 cc. of the suspected water. If the 0.1 cc. tubes show gas, we have reason to assume that the water contained at least 10 organisms per cc. If only 10-cc. tubes showed gas—those with less amounts not having gas—we would be in a position to state that the water contained the organisms in quantities of 10 cc., but not in quantities of 1 cc. or less. Many authorities regard water as suspicious only when members of the *coli-aerogenes* group are present in quantities of 10 cc. or less, water of good quality frequently showing their presence in quantities of 100 to 500 cc.

It is generally accepted that if a water shows the presence of these organisms in quantities of 1 cc. or less, it should be regarded as suspicious.

A medium formerly recommended as yielding very low errors in carrying out the quantitative presumptive tests is the lactose bile, made by adding 1% of lactose and 1% of peptone to ox bile. Fermentation tubes of the media showing gas may be considered as very probably containing members of the *coli-aerogenes* group. The error with this method is reported to be only 11%, while with glucose fermentation tubes the error is more than 50%. Gas formation is usually shown in forty-eight hours, but it is advisable to continue the incubation for seventy-two hours. These presumptive tests are chiefly of value in highly contaminated waters. Even with this method plates should be made. Lactose bouillon, however, is now generally used instead of lactose bile.

3. As the *coli-aerogenes* group and sewage streptococci ferment lactose with the production of acid and hence produce pink colonies on lactose litmus agar, much information can be obtained from the proportion existing between the number of pink colonies and those not having such a color. Waters of fair degree of purity rarely give any pink colonies.

A commission composed of eminent American bacteriologists and sanitarians have recommended the following as maximum limits of bacteriological impurity:

1. The total number of bacteria developing in standard agar plates, incubated twenty-four hours at 37°C., shall not exceed 100 per cc.: Provided that the estimate shall be made from not less than two plates, showing such numbers and distribution of colonies as to indicate that the estimate is reliable and accurate.

2. Not more than one out of five 10-cc. portions of any sample examined shall show the presence of organisms of the *coli-aerogenes* group.

3. It is recommended, as a routine procedure, that in addition to five 10-cc. portions, one 1-cc. portion, and one 0.1-cc. portion of each sample examined be planted in a lactose-peptone broth fermentation tube, in order to demonstrate more fully the extent of pollution in grossly polluted samples.

Determination of Bacteria Present in Water.—The American Public Health Association has in its "Standard Methods," 1925 edition, recommended the following tests for the presence of members of *coli-aerogenes* group and for the differentiation of faecal from nonfaecal members of the same group.

Tests for the presence of members of the coli-aerogenes group.—It is recommended that the *coli-aerogenes* group be considered as including all Gram-negative nonspore-forming bacilli which ferment lactose with gas formation and grow aerobically on standard solid media.

The formation of 10% or more of gas in a standard lactose broth fermentation tube within 24 hours at 37°C. is presumptive evidence of the presence of members of this group, since the majority of the bacteria which give such a reaction belong to this group.

The appearance of aerobic lactose-splitting colonies on eosin methylene blue or Endo plates made from a lactose broth fermentation tube in which gas has formed confirms to a considerable extent the presumption that gas formation in the fermentation tube was due to the presence of members of the *coli-aerogenes* group.

To complete the demonstration of the presence of organisms of this group, it is necessary to show that one or more of these aerobic plate colonies consist of nonspore-forming bacilli which, when inoculated into a lactose-broth fermentation tube, form gas.

It is recommended that the standard tests for the *coli-aerogenes* group be either (a) the *Presumptive*, (b) the *Partially Confirmed*, or (c) the *Completed* test as hereafter defined, each test applicable under the circumstances specified.

A. Presumptive Test

1. Inoculate a series of lactose broth fermentation tubes with appropriate graduated quantities of the water to be tested. In every fermentation tube there must always be at least twice as much medium as the amount of water to be tested. When necessary to examine larger amounts than 10 cc. as many tubes as necessary shall be inoculated with 10 cc. each.

2. Incubate these tubes at 37°C. for 48 hours. Examine each tube at 24 and 48 hours, and record gas formation. The records should be such as to distinguish between:

(a) Absence of gas formation.

(b) Formation of gas occupying less than 10% of the inverted vial.

(c) Formation of gas occupying more than 10% of the inverted vial.

More detailed records of the amount of gas formed, though desirable for purposes of study, are not necessary for carrying out the standard tests prescribed.

3. The formation within 24 hours of gas occupying more than 10% of the inverted vial in the fermentation tube constitutes a *positive presumptive test.*

4. If no gas is formed in 24 hours, or if the gas formed is less than 10%, the incubation shall be continued to 48 hours. The presence of gas in any amount in such a tube at 48 hours constitutes a *doubtful test*, which in all cases requires confirmation.

5. The absence of gas formation after 48 hours' incubation constitutes a *negative test.* (An arbitrary limit of 48 hours' observation doubtless excludes from consideration occasional members of the *coli-aerogenes* group which form gas very slowly, but for the purposes of a standard test the exclusion of these occasional slow gas-forming organisms is considered immaterial.)

B. Partially Confirmed Test

1. Make one or more Endo or eosin methylene blue plates from the tube which shows gas formation from the smallest amount of water tested. Make transfers as soon as possible after gas formation occurs. (For example, if the water has been tested in amounts of 10 cc., 1 cc., and 0.1 cc., and gas is formed in 10 cc., and 1 cc., not in 0.1 cc., the test need be confirmed only in the 1 cc. amount.)

2. Incubate the plates at 37°C., 18 to 24 hours.

3. If typical colonies have developed upon the plate within this period, the confirmed test may be considered positive.

4. If, however, no typical colonies have developed within 24 hours, the test cannot yet be considered definitely negative, since it not infrequently happens that

members of the *coli-aerogenes* group fail to form typical colonies on Endo or eosin methylene blue plates, or that the colonies develop slowly. In such case, it is always necessary to complete the test as directed under "C" 2 and 3.

C. Completed Test

1. From the Endo or eosin methylene blue plates made as prescribed under "B" fish at least two typcal colonies, transferring each to an agar slant and lactose broth fermentation tube.

2. If no typical colonies appear upon the plate within 24 hours, the plate should be incubated another 24 hours, after which at least two of the colonies considered most likely to be organisms of the *coli-aerogenes* group, whether typical or not, shall be transferred to agar slants and lactose broth fermentation tubes.

3. The lactose broth fermentation tubes thus inoculated shall be incubated until gas formation is noted, the incubation not to exceed 48 hours. The agar slants shall be incubated at 37°C. for 24 hours, when a microscopic examination shall be made of at least one culture, selecting one which corresponds to one of the lactose broth fermentation tubes which has shown gas formation.

The formation of gas in lactose broth and the demonstration of Gram-negative nonspore-forming bacilli in the agar culture shall be considered a satisfactory completed test, demonstrating the presence of a member of the *coli-aerogenes* group.

The absence of gas formation in lactose broth or failure to demonstrate Gram-negative nonspore-forming bacilli in a gas-forming culture constitutes a negative test.

Note.—In order that tests for *coli-aerogenes* group may have quantitative significance, the following general principles and rules should be observed:

Ordinarily not less than three portions of each sample should be tested, the portions being even multiples or fractions of a cubic centimeter, i.e., 10 cc., 1 cc., 0.1 cc., 0.01 cc., etc. The largest amount used should give a positive test (except when negative in 10 cc.) and the smallest a negative test.

As recent work seems to indicate that the *coli-aerogenes* group as defined above consists of organisms of both faecal and nonfaecal origin, great care must be used in judging the sanitary quality of a water solely on bacteriological examination for members of this group.

Differentiation of faecal from nonfaecal members of the coli-aerogenes group.—The following tests are tentatively recommended for distinguishing faecal from nonfaecal strains of organisms in the *coli-aerogenes* group. At the present time our knowledge is not sufficiently complete to warrant the adoption of any single test or group of tests. The procedures are presented with the hope that positive evidence will gradually accumulate.

Methyl red test.—The peptone medium has been described on page 37. Inoculate 10 cc. portions of this medium. Incubate at 37°C. for 4 days. Use 5 cc. of culture for methyl red test and 5 cc. for Voges-Proskauer reaction.

The methyl red indicator solution is prepared by dissolving 0.1 gram methyl red in 300 cc. alcohol and then diluting to 500 cc. with distilled water. Five drops of this solution are then added to 5 cc. of each culture. A distinct red color is recorded as methyl red +, a distinct yellow color as methyl red −, and intermediate colors as ?. The *Voges-Proskauer test* has been described on page 37.

Uric acid test.—Dissolve 5 Gm. sodium chloride, 0.2 Gm. magnesium sulphate, 0.1 Gm. calcium chloride, 1 Gm. dipotassium hydrogen phosphate, 30.1 Gm. glycerol and 0.5 Gm. uric acid in 1000 cc. distilled ammonia-free water. Tube and sterilize as usual. Incubate cultures for 4 days at 37°C. and record growth as + or −.

Sodium citrate test.—Dissolve 1.5 Gm. sodium ammonium phosphate (microcosmic salt), 1 Gm. potassium dihydrogen phosphate, 0.2 Gm. magnesium sulphate and 2.5 to 3 Gm. sodium citrate (crystals) in 1 liter distilled water. Incubate cultures for 4 days at 37°C. and record growth as + or −.

The Coli-aerogenes Group.—The diagnostic characteristics considered important by the American authorities in reporting the colon bacillus are:

1. Typical morphology; nonsporing bacillus; relatively small and often quite thick.
2. Motility in young broth cultures. (This is at times unsatisfactory, as some strains of the colon bacillus do not show it even in young bouillon cultures.)
3. Gas formula in dextrose broth. Of about 50% of gas produced, one-third should be absorbed by a 2% solution of sodium hydrate (CO_2). The remaining gas is hydrogen. (Later views indicate that the gas formula is exceedingly variable and should not be depended upon. To carry out this test one fills the bulb of a fermentation tube with the caustic soda solution, holding the thumb over the opening, or inserting a rubber stopper, the bouillon culture and the soda solution are mixed by tilting the fermentation tube to and fro. The total amount of gas is first recorded and then that remaining after the CO_2 has been absorbed is reported as hydrogen.)
4. Nonliquefaction of gelatin.
5. Fermentation of lactose with gas production.
6. Indol production.
7. Reduction of nitrates to nitrites.

To these may be added the acidifying and coagulation of litmus milk without subsequent digestion of the casein. The production of gas and fluorescence in glucose neutral red bouillon is also a very constant function of the colon bacillus. *B. aerogenes* is similar to *B. coli* with the exception of nonmotility, formation of gas in starch media (bubbles on potato slant) and frequent lack of indol production. It is often, especially in milk cultures, provided with a capsule and rarely forms chains. It is a member of the Friedländer group but differs from the typical pneumobacillus by producing acid and gas in lactose broth and by its coagulation of milk.

B. coli anacrogenes also is similar to *B. coli* but does not produce gas in glucose and lactose. This organism is not usually recognized by American authorities but I have found on Endo plates an organism showing a red colony with metallic luster which failed to produce gas in either glucose or lactose.

NOTE.—The reduction of neutral red with a greenish-yellow fluorescence is very striking and has been suggested as a test for the colon bacillus. Many other organisms, especially those of the hog cholera group, have this power. It is convenient, however, to color glucose bouillon with about 1% of a 0.5% solution of neutral red.

On the plates made for the detection of colon bacillus may be found certain organisms which have origin in faecal contamination. The more important of these are those of the paratyphoid, cloacae and proteus groups. In addition, the *B. faecalis alkaligines* has not rarely been isolated. Among natural water bacteria there may be present either the liquefying or the nonliquefying *B. fluorescens*. These colonies have a yellowish-green fluorescence.

Certain chromogenic cocci and bacilli are found in uncontaminated waters as *B. indicus* or *B. violaceus*. From surface washings we obtain certain soil bacteria as *B. mycoides*, *B. subtilis*, *B. megaterium*. One of the higher bacteria which shows long threads, *Cladothrix dichotoma*, is common, and is characterized by a brown halo around its gelatin-plate colony.

Freezing kills most of the bacteria in water. Less than 1% of typhoid organisms inoculated into water survive a week of freezing. Ice that has been stored for 4 or 5 months is bacterium-free.

Isolation of the Typhoid Bacillus from Water.—This is probably the most discouraging procedure which can be taken up in a laboratory. Only the most recent reports of such isolation from water supplies, which have been verified by immunity reactions, can be accepted and of these the number of instances is exceedingly small. Owing to the long period of incubation of the disease, the typhoid organisms may have died out before the outbreak of an epidemic suggests the examination of the water supply.

There have been various methods proposed for the detection of the *B. typhosus* in water. A method which would offer about as reasonable a chance of success as any other would be to pass 2 or 3 liters of the water through a Berkefeld filter; then to take up in a small quantity of water all the bacteria held back by the filter. Then plate out on lactose litmus agar and examine colonies which do not show any pink coloration. The dysentery bacillus has about the same cultural characteristics as the typhoid one, so that it is important to note motility. If from such a colony you obtain an organism giving the cultural characteristics of *B. typhosus*, carry out agglutination and preferably bacteriolytic tests as well. Some strains of typhoid, especially when recently isolated from the body, do not show agglutination.

The Conradi-Drigalski, the malachite-green, and various caffeine-containing plating media have been highly recommended.

Isolation of the Cholera Spirillum from Water.—The method proposed by Koch in 1893 does not seem to have been improved upon by later investigators. To 100 cc. of the suspected water add 1% of peptone and 1% of salt. Incubate at 38°C. and at intervals of eight, twelve, and eighteen hours examine microscopically loopfuls taken from the surface of the liquid in the flask. So soon as comma-shaped

organisms are observed, plate out on agar. The colonies showing morphologically characteristic organisms should be tested as to agglutination and bacteriolysis. Inasmuch as the true cholera spirillum shows a marked cholera-red reaction it is well to inoculate a tube of peptone solution from such a colony and add a drop of concentrated sulphuric acid after incubating for eighteen hours. The rose-pink coloration is given by the cholera spirillum with the acid alone—the nitroso factor in the reaction being produced by the organism.

Bacteriological Examination of Milk

A bacterial milk count is of comparatively little value as showing whether a milk is dangerous or not. As a matter of fact, a milk which contains several million of bacteria per cc. might be less dangerous than one containing only a few thousand, especially if in the latter there were present numerous liquefiers and gas-producers. There is, however, one point of importance in connection with the quantitative estimation of bacteria in milk, and that is the fact that in order to keep the development of the bacteria within the limits of 10,000 to 50,000 per cc., it is necessary that the requirements of cleanliness in milking and the rapid cooling of the milk after obtaining it and the keeping of the temperature below 50°C. be rigidly observed. If a milk has a high count it shows some error in the handling of the milk. Anderson has found that top milk contains from 10 to 500 times as many bacteria as bottom milk. Centrifugally separated cream contains more bacteria than that raised by gravity. In making a quantitative bacteriological examination, the principle is the same as with water.

Cultural Method of Counting.—Make a known dilution of the milk with sterile water; add definite quantities of this diluted milk to tubes of melted agar or gelatin, and pour into plates. The diluted milk may also be delivered in the center of the plate and the melted agar or gelatin poured directly on it, mixing thoroughly. Always shake the bottle well before taking sample.

Example.—Added 1 cc. of milk to 199 cc. of sterile water in a large flask (500 to 1000 cc.). After shaking thoroughly, take 1 cc. of this 1 : 200 dilution and add it to 99 cc. of sterile water. Shaking thoroughly, we have a dilution of 1 : 20,000. Of this we added 0.5 cc. to a tube of gelatin or agar. After incubation the plate showed 75 colonies. Therefore the milk contained in each cc. $75 \times 2 \times 20{,}000$ (dilution) = 3,000,000—the number of bacteria in each cc. of milk.

Lactose litmus gelatin or agar is to be preferred in milk work, as the normal lactic acid bacteria produce reddish colonies which are very striking. A standard easily attained for high-grade, certified milk would be 5000 to 10,000 per cc.

In the qualitative examination of milk, many dairies employ the fermentation tube, any organism producing gas being considered undesirable. Again, liquefying organisms, as shown by the presence of such bacteria in the gelatin plates, are evi-

dence of probable contamination by faecal bacteria. A question which seems difficult to decide is as to the general nature of the so-called normal lactic acid bacteria of milk. Some describe them as very short, broad bacilli with very small colonies, fermenting lactose with the formation of lactic acid. Others consider that the streptococci are the organisms which are concerned with the normal fermentative changes. In examining specimens of milk considered the best on the market, I have repeatedly found the small red colonies on lactose litmus agar to be either Gram-positive streptococci or streptobacilli.

Microscopic Method of Counting.—In the method of ascertaining the number of bacteria present in a given quantity of milk by counting the colonies which develop on culture, sources of error are present in that many organisms may have died or be unable to grow on the ordinary media usually employed. The Breed method, devised to avoid these defects of the cultural method, makes possible a direct count of the number of organisms present.

Technique: Place over a white background a piece of black paper out of which has been cut an area 1 cm. square. A clean slide is laid on the paper, and 0.01 cc. of milk deposited on it directly above the aperture. The milk is then smeared out evenly with a clean needle to cover an area of 1 sq. cm. as measured by the aperture beneath. Another method is to use a slide on which a square centimeter has been directly ruled with a diamond point. Dry the smear held high above a small flame, excessive heat being avoided else the film will crack and peel off in the later steps of the technique. Immerse the slide for two and a half minutes in a Coplin jar filled with xylol to remove fat; drain, and immerse in acetone for 2 or 3 minutes. Then stain in a fresh aqueous solution of methylene blue until overstained. Next wash in water and decolorize for a minute or so in alcohol. After slide is dried, place on the mechanical stage and proceed to estimate the number of bacteria.

In order to do this, the area of the microscopic field must be known. Using a $\frac{1}{12}$-inch objective and a 5 × ocular, it will be found that by adjusting the tube length the diameter of the field can be made to coincide with a line formed by the sides of 4 small squares of the haemacytometer ruling, the radius of the circular field then being 2 × the side of a small square (0.05 mm.) or 0.1 mm. The total area then included in the circular field (πr^2) is $3.1416 \times (0.1)^2$ sq. mm. or $\frac{1}{3183}$ sq. cm. Hence each field will contain $\frac{1}{3183}$ of the total number of bacteria present in the square centimeter covered by the 0.01 cc. of milk. The average of the counts of a large number of fields in different parts of the stained milk smear having been obtained, this average is multiplied by 3183 to ascertain the number of bacteria in the entire smear. Since this method is not precise, it suffices to multiply by 3000 instead of by 3183. As only $\frac{1}{100}$ of a cubic centimeter of milk was deposited on the slide, the last figure is multiplied by 100 to obtain the number of bacteria in 1 cc.

For example: If the number of bacteria per field averaged 10, we would have $10 \times 3000 \times 100 = 3{,}000{,}000$ bacteria per cubic centimeter of the milk examined. With milks having a low count a special ocular micrometer may be used.

The Reductase Test.—This test is also known as the reduction test or methylene blue test. It is based on the fact that the color imparted to milk by a small quantity of methylene blue will disappear more or less quickly. The rate of disappearance depends almost entirely on the number of bacteria present, if other factors such as temperature are kept constant. Specially prepared tabloids of methylene blue are

recommended for preparing the reagent, but a suitable stock solution may be prepared by dissolving 1.1 Gm. of the dry dye in 500 cc. distilled water. One cc. of this solution is diluted to 40 cc. with water. The resulting solution will be one part of the dye in 20,000 parts of water. It is advisable to heat the solution to 100°C. a few minutes before use.

For the test the milk should be collected the same way as for any bacteriological examination. Ten cc. of milk is placed in a test tube and 1 cc. of the reagent rapidly added. Blow the solution from a pipette into the milk, so the dye will be distributed throughout the entire depth. The color imparted to the milk is a robin's egg blue. Tubes are then placed in a water bath at 37°C. or in an incubator. The end point is taken when the blue color has disappeared and the milk has regained its normal color. In certain European countries where the test is widely used the color of the upper ¼ of the tube is neglected in reading the test, especially in case the color disappears in an uneven manner.

Comparisons between the reduction time and the plate culture count have been made by a number of investigators. An exact agreement between reduction time and plate count cannot be expected. It is known that there are bacteria present in many samples of milk that are not determined by the ordinary culture method and these bacteria undoubtedly function in the reductase test. The field of the reductase test is in making a rapid survey of the milk supplied, in exercising some control where laboratory facilities and trained help are not available. There is every reason to believe that the detection of poor quality milk can be accomplished as accurately by this test as any of the other methods.

The following scheme of classification is used in Scandinavian countries where the test is widely employed in milk control work:

Class I. Good milk.—Not decolorized in 5½ hrs., containing as a rule less than one-half million bacteria per cc. (As determined by the plate method.)

Class II. Milk of fair average quality.—Decolorized in less than 5½ hrs., but not less than 2 hrs., containing as a rule one-half to four million bacteria per cc.

Class III. Bad milk.—Decolorized in less than 2 hrs., but not less than 20 minutes, containing as a rule, from four to twenty million bacteria per cc.

Class IV. Very bad milk.—Decolorized in 20 minutes or less, containing as a rule twenty million or more bacteria per cc.

Acid Producing Organisms.—Shippen considers the chief organism concerned in the souring of milk to be *B. güntherii*, but notes that it is the same organism as *S. lacticus*. All authors note the difficulty of deciding whether the morphology is coccal or bacillary. McGuire found these organisms almost constantly present in the dung of cows. The organisms he obtained from cow dung were chiefly members of the coli-aerogenes group and *S. lacticus*.

Of the acid-forming bacilli in milk we have (1) the *B. lactis acidi* group. These are oval cells about 0.9 microns by 0.6 microns, often occurring in chains. They are Gram-positive and nonmotile. They may be the same as *Streptococcus lacticus* of

Kruse. They curdle milk with a homogeneous clot—this being due to the fact that they do not produce gas in lactose media. (2) The *B. aerogenes* group. These are gas-producers. (See under Water.) (3) The *B. bulgaricus* group. In connection with the organisms present in the tablets used for treating milk to produce lactic acid for the treatment of intestinal disorders, and considered to be normal lactic acid bacteria, I have found both streptococci and bacilli. These have agreed, however, in not producing gas in either lactose or glucose fermentation tubes.

I have often found the commercial fluid cultures sterile, the great acidity produced by *B. bulgaricus* causing this. Fresh tubes may show an acidity of +12 or about 10 times that of ordinary culture media.

The organism upon which special stress is laid among these so-called lactic acid-producers is the *B bulgaricus*. This is a large, nonmotile organism with square ends like anthrax. It often occurs in long chains and does not possess spores. It is Gram-positive and often shows metachromatic granules like those of the diphtheria bacillus. Colonies show in forty-eight hours which resemble streptococcus ones, but are more contoured on the surface. Magnified the colonies resemble young mould colonies. It grows better on milk agar plates than on whey agar plates. The opacity of the milk agar plate is but a slight objection. It produces a deep vivid pink in litmus milk, while milk streptococci cause only a light pink. It produces a very large amount of acid (3%). Little or no growth on ordinary laboratory media or below 20°C. (Opt. temp. 42°C.)

Heinemann states that it occurs normally in human faeces and variously fermented milk—also in gastric juice when HCl is absent. To isolate, put milk or faeces into a broth containing 0.5% acetic acid and 2% glucose. Transfer to litmus milk after twenty-four hours and from such tubes plate out on milk serum agar (coagulate boiling milk with a few drops of acetic acid, filter and add 1% peptone, 2% glucose and 1.5% agar).

As these bacteria grow in very acid media the term acidophilous is applied to them. It was supposed that they were peculiar to certain fermented milks as matzoon and yogurt. Hastings has shown the group to be present in milk in the United States and considers the source to be the alimentary tract of cows.

Butyric acid fermentation.—The organisms concerned are as a rule anaerobes and may be either pathogenic or saprophytic. Alcoholic fermentation of milk, as in kefir, is probably due to yeasts. Various chromogenic bacteria may cause blue, red or yellow milk, and bitter milk is probably caused by yeasts.

Milk Leukocytes.—Another source of information as to the quality of a milk may be derived from a study of the number of leukocytes or pus cells contained in 1 cc. of milk. It must be understood that cellular elements which differ only slightly from true pus cells may be found in the milk of healthy cows and may be found in great numbers. Statements have been made that such cells are neither amoeboid nor phagocytic.

The Doane-Buckley method is probably the most accurate. In this you throw down the cellular contents of 10 cc. of milk in centrifuge revolving about 1000 times a minute for ten to twenty minutes. Then remove supernatant milk and add 0.5 cc. of Toisson's solution to the sediment. Instead of Toisson's solution I use Gram's iodine solution which brings out the leukocytes equally well and gives a cleaner preparation. You thus have the leukocytes of 10 cc. contained in 0.5 cc. (concentrated 20 times). Make a haemacytometer preparation as for blood and find the average number of cells for each square millimeter. Then multiply this by 10 to get the number of cells in a cubic millimeter. As a cubic millimeter is 1000 times smaller than a cubic centimeter, you multiply the number per cubic millimeter by 1000. Then, as the milk was concentrated 20 times, you divide by 20. (If it were diluted 20 times, you would multiply by 20.)

Example.—Found an average of 50 cells per square millimeter. This would make 500 per cubic millimeter, and 500,000 per cc.; then 500,000 divided by 20 would give 25,000.

There is no agreement as to a standard for allowable leukocytes. Even in apparently healthy animals they may exceed 100,000 per cc. Doane has suggested 500,000 per cc. as a preferable limit.

The smear methods for determining the number of leukocytes present do not compare in accuracy with the volumetric ones. It is important, however, from a standpoint of examining for tubercle bacilli, etc., as well as for recognition of leukocytes, to deposit the sediment from a centrifuge tube, taken up with a capillary bulb pipette, on a glass slide. Smear out slightly and then when dry fix with a mixture of ether and absolute alcohol. Flood with ether to get rid of remaining fat and stain by Gram's method or by acid-fast staining. The sediment in the tube gives an idea of the dirt in the milk. A milk which shows visible dirt in the bottle or when passed through cotton should be condemned.

To summarize, we may state that the bacterial count is an indicator of the care used in handling the milk while the presence of certain harmful bacteria (qualitative examination) or numerous pus cells indicates disease in the cow.

During 1912 severe epidemics of sore throat due to a streptococcus, *S. epidemicus*, were traced to milk of cows having probably suffered from mastitis. In Baltimore the milk had been pasteurized by the flash method which indicates the unreliability of this process. It has now been determined that these streptococci were of human and not bovine origin, milkers with streptococcic infections contaminating the cow's udder. While experiments have shown that certain streptococci may resist the pasteurizing temperature (145°F. for thirty minutes) yet with pathogenic streptococci of human source such a temperature has been entirely effective. This would show that milk properly pasteurized is safe from a standpoint of causing streptococcal sore throat.

Pasteurization of Milk.—The objections to this method of preserving milk have been (1) that the lactic acid bacteria, which have been by some credited with antagonism to harmful bacteria, would be destroyed by pasteurization, (2) the more rapid

development of bacteria in milk that has been pasteurized, (3) interference with nutritive qualities and (4) pasteurized milk does not show its deterioration as does unpasteurized milk, thus failing to give a clue as to the age of the milk.

The United States Bureau of Animal Industry in studying this important phase of the milk question has grouped the milk bacteria into three classes: (*a*) Acid-forming, (*b*) putrefactive (liquefying) and (*c*) inert bacteria. In their investigations it was found that many acid-forming bacteria withstood temperature as high as 168°F., so that pasteurized milk was soured just as is raw milk, but more slowly. They found that pasteurized milk showed fewer putrefactive bacteria than raw milk, so that even should it be a fact that injurious toxins were produced by spore-bearing putrefactive organisms the development of such organisms would be even less in pasteurized milk.

The statement so often advanced that bacteria develop more rapidly in pasteurized milk than in raw milk was proved fallacious.

It was recommended that holding the milk for thirty minutes at 145°F. was a far better method of pasteurizing than quickly bringing the milk to a temperature of 185°F. (flash method). In one flash method the milk is passed through the tubes which raise the temperature to 170°F. and then through cooling tubes, the process taking only 5 or 6 seconds. The taste of milk pasteurized by the holding system is unaffected which is not true of the flash system. All admit the great value of the killing of important pathogens (typhoid, cholera, streptococci, etc.). The diseases which should be particularly kept in mind in connection with disease transmission are tuberculosis, septic sore throat, bacillary dysentery, typhoid and paratyphoid fevers, diphtheria, scarlet fever and cholera. Foot-and-mouth disease is also transmitted to children by milk. The milk of infected goats and fresh cheese made therefrom may transmit undulant fever. For Calcium Loss, see page 770.

Bacteriological Examination of Air

In Paris a cubic meter of air was found to contain the following number of organisms:

Suburbs.—Winter.	145 moulds,	170 bacteria.
Summer.	245 moulds,	345 bacteria.
City Hall.—Winter.	1345 moulds,	4305 bacteria.
Summer.	2500 moulds,	9845 bacteria.

Air of hospitals, especially after sweeping, may contain 50,000 bacteria per cubic meter. There does not seem to be any particular relation between the amount of carbon dioxide in air and the bacterial content.

Petri's Rough Method.—Exposure of a lactose litmus agar plate (capacity 100 sq. cm.) for five minutes will give the number of organisms present in 10 liters of air. Multiply by 100 for 1 cu. m. On the lactose litmus agar plate staphylococci and streptococci show as bright red colonies.

Sedgwick-Tucker Sterile Granulated Sugar Method.—Sterilize aerobioscope and introduce granulated sugar on support. Again sterilize (not over 120°C. in dry-air sterilizer). Allow a given quantity of air to pass through; then shake the sugar into wide part of aerobioscope. Now pour in 10 or 15 cc. of melted gelatin (40°C.) to dissolve sugar. Roll tubes as for Esmarch roll cultures, and incubate at room temperature. To draw air through the aerobioscope, connect the small end with a piece of rubber tubing which is attached to a tube in the stopper of an aspirating bottle. Having poured a definite quantity of water into the aspirating bottle, allow the water to run out. The same quantity of air will be drawn through the sugar of the aerobioscope as the amount of water passing out of the aspirating bottle. The bacteria and moulds are caught by the sugar.

Example.—Passed 10 liters of air through the aerobioscope. The bacteria in this quantity of air showed 75 colonies when incubated at 20°C. The unit being 1 cu. m. or 1000 liters, we have only obtained the bacteria of one hundredth of the unit. Hence multiplying 75 by 100 gives 7500 bacteria as present in 1 cu. m. of the air examined.

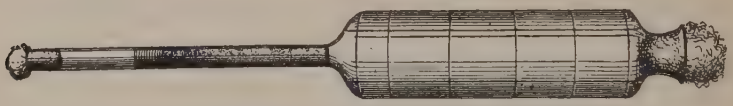


FIG. 59.—Sedgwick-Tucker aerobioscope. (*Mac Neal.*)

A very satisfactory method is to take a test tube containing 5 cc. of sterile water and having a rubber stopper with two perforations, one for a long piece of glass tubing which dips down into the water and one for a short piece which is connected with the aspirating bottle by rubber tubing.

The air to be examined is drawn through the long tube and its bacterial or mould content is caught in the water. By plating 1 cc., which would represent one-fifth of the total count for the amount of air aspirated, we can easily calculate the content for a cubic meter.

In comparing the results with the aerobioscope with those obtained by exposing a plate as in Petri's method for ten instead of five minutes, it was found that the latter was sufficiently in accord to make it a satisfactory approximate quantitative method. The simplicity and ease of access to the colonies developing on it make it preferable when the air of operating-rooms or hospital wards is to be examined.

Organisms in Air.—The two groups of organisms usually found in air are: 1. Bacteria and 2. moulds. Moulds (spores) may be carried by currents of air; bacteria, however, are generally carried about by particles of dust or finely divided liquids (spray). Of the fungi ordinarily obtained in examinations of the air the blue-green mould or the red yeast are the most common. *B. subtilis* and sarcina types of cocci are the most common bacterial colonies found upon exposed plates. Sewer air is as a rule free from bacteria, due probably to the fact that bacteria tend to adhere to moist

surfaces. The importance of Flügge's droplet method of contamination of the air of a room is brought out in the discussion of infection with pneumonic plague. This is by some considered as a method in the transmission of tuberculosis. It is of importance in the spread of type I and II pneumonias but experiments fail to show it of importance in the spread of influenza.

Ventilation.—Recent studies in ventilation emphasize the importance of temperature, relative humidity and air motion as the important features and minimize the harmful effects of increased CO_2 content, "noxious emanations" and bacterial contamination.

CHAPTER XII

PRACTICAL METHODS IN IMMUNITY

That power by means of which disease organisms are prevented from gaining a foothold in the animal body or their harmful products neutralized or they themselves destroyed is termed immunity. In the main, the question of immunity hinges on the powers of resistance of the human body and the aggressiveness or virulence of the invading organism.

It must always be kept in mind that immunity is only relative; thus the fowl, which is practically immune to tetanus, may be made to succumb by reducing its resistance by refrigeration or by increasing the amount of poison introduced. The lack of susceptibility which the fowl has to tetanus or which man has to many diseases of animals is best termed *inherent* or *inborn immunity*, and is at present a subject only of theoretical interest. When immunity to a given disease is obtained as a result of an attack of the disease in question or by laboratory methods of inoculation, this is termed properly an *acquired immunity*, and in the former case is a *naturally acquired immunity* and in the second an *artificially acquired immunity*.

Immunity then may be divided into that which is *inherent* and that which is *acquired*. Inherent immunity is such as is observed in the resistance of Algerian sheep to anthrax (ordinary sheep are very susceptible) or laboratory animals to leprosy or man to many of the diseases of domesticated animals. Acquired immunity may be brought about naturally as by an attack of a disease or artificially by laboratory measures.

Antibodies.—As a result of an attack of a disease, which may be regarded as accidentally acquired, or in response to the stimulus of the injection of the causative organism or its products, we have developed in the man so infected or injected certain specific properties antagonistic to that organism, which are usually demonstrable in the blood serum or other body fluids, and to which we apply the terms agglutinating power, precipitating power, opsonic power, or bacteriolytic power. The general term *antibody* is also applied.

All four powers or antibodies may be present together in equal or in varying degree or one or more may be absent. By *agglutinating power* we mean that which causes evenly distributed organisms to come together and form clumps. By *precipitating power* we mean the ability of a serum possessing it to cause precipitates

in a clear bouillon filtrate of the specific bacterium. Such antibodies are called precipitins or coagulins. By *opsonic power* we infer the presence of an antibody which so alters the resistance of bacteria that the phagocytes ingest them. By *bacteriolytic power* we mean that which brings about disintegration or lysis of the specific organisms.

The organism which causes the disease or which is used in inoculation for the production of immunity is termed the *specific organism.*

Artificially Acquired Immunity.—Of the different kinds of immunity only artificial immunity will be considered. This may be obtained in two ways, actively and passively:

1. *Active immunity.*—As the result of the activity of the cells of a man or animal following the injection of bacteria or their products, antibodies are formed which neutralize the toxins (antitoxins) or bring about lysis of the specific bacteria (bacteriolysins). These antibodies, which are supposed to be thrown off (free receptors) from those body cells which have suitable fixation powers for the invading toxin molecule or bacterium, may remain potential for months or years and so confer a more or less enduring immunity.

These fixation points are known as *cell receptors* and are intended for the assimilation of various foodstuffs by the cell. If destroyed by the toxin or bacterium they are reproduced in great excess by nature so that the producing cell can no longer contain the overproduction but casts them off into the body fluids. This excess production by nature over replacement needs is known as Weigert's hypothesis.

Not only may bacteria act in this way but certain protozoa and foreign cells, such as red cells or various parenchymatous cells, when injected, give rise to antagonistic substances which act as factors in their destruction—haemolysins for red cells, cytolysins for different parenchymatous cells. Such methods produce "*active immunity.*"

Antigen.—The substance which is injected and in reaction to which antibodies are produced is called an antigen. It is generally considered that proteins alone (animal or plant) have antigenic power.

2. *Passive immunity.*—When we take the serum of a man or animal immunized actively and inject it with its contained antibodies into a second animal or man, we confer an immunity on the second animal; but as his cells take no active part in the production of the immunity, but are only passive, we term this immunity, "*passive immunity.*"

If this serum which is introduced in passive immunity neutralizes only the toxic products of the infecting bacteria, we term it antitoxic passive immunity and designate the immune serum as *antitoxic serum.* If it destroys the organism, we call it *antimicrobic serum,* and the immunity, antimicrobic passive immunity. Some immune sera are both antitoxic and antimicrobic.

Toxins.—It is well to remember that some organisms produce a soluble or extracellular toxin which is given off while the bacterium is alive; and in other instances the toxin is intracellular and is given off only when the bacterium disintegrates; consequently, an antimicrobic serum may cause the liberation of endotoxin. Diphtheria, tetanus, or botulism antisera are instances of antitoxic sera, while practically all others are antimicrobic. The antidysentery serum against Shiga strains seems to have antitoxic power. *B. pyocyaneus* and certain of the pathogenic anaerobes besides *B. botulinus* also produce a soluble toxin.

A true toxin.—The term toxin, strictly speaking, is applicable only to such bacterial poisons as (1) require a period of incubation before being capable of manifesting toxic symptoms and (2) can produce antitoxins. These antitoxins are produced only by the soluble toxins or exotoxins and never by endotoxins. Our knowledge of endotoxins is rather uncertain, and the recent demonstration by Zinsser of nonspecific toxic substances, obtained from various bacteria not capable of producing soluble toxins, would indicate that such toxic effects might have been considered as due to specific endotoxins. The true toxins with the possible exception of that of *B. pyocyaneus* are destroyed by temperatures varying between 60° and 70°C. while endotoxins usually withstand 100°C. As a practical point, boiling food kills the soluble toxin of *B. botulinus* but does not destroy the toxic products (endotoxins?) of the Gärtner bacillus.

Antitoxic Sera.—There is but one factor to consider in an antitoxic serum and that is the protoplasmic particles which are thrown off from the cell in response to the injury incident to the attack upon the cell by the toxin particles. This free particle in the circulation represents the entire mechanism of antitoxic immunity. It is capable of uniting with the toxin molecule and neutralizing its toxic power; or, in other words, the antitoxic particle, by occupying the combining end of the toxin, deprives it of its means of effecting union with the cell.

For further discussion of toxins and antitoxins see under diphtheria, tetanus, botulism, and pyocyaneus infections.

Antimicrobic Sera.—In antimicrobic sera we have two factors to consider.

The first, generally referred to as the *amboceptor* or *immune body*, is a protoplasmic particle quite similar to the antitoxin molecule. Although it is a specific product of the body cells, elaborated as a means of defense against the action of a particular kind of bacterium or foreign cell, yet of itself it is incapable of injuring the bacterium in response to whose attack it was produced. It is thermostable, withstanding a temperature above 56°C.

The second factor in the bacteriolysis of the specific bacterium, or the haemolysis of the specific foreign cell, is something called the *complement*, or alexine, or cytase (Metchnikoff). It is normally present in the serum of every animal, and is capable of disintegrating a foreign cell or bacterium, provided it can have access to the cell or

bacterium through an intermediary amboceptor (hence the amboceptor is sometimes called an intermediary body).

The complement cannot act upon and destroy an invading bacterium or cell unless the amboceptor is present to make the necessary connection. The complement is destroyed by a temperature of 56°C., so that, if we heat the serum from an immune animal to 56°C., the complement it naturally contains is destroyed, and the amboceptor it contains, which is not injured by such a temperature, is incapable of destroying bacteria or cells, unless we replace the complement, which has been destroyed, by fresh complement. This is done experimentally by adding the serum of a nonimmunized animal which contains the complement, but no specific immune body (amboceptor), to the heated serum. This is termed "activating," and a serum so treated is said to be "*activated*." When an immune serum has been heated to 56°C., it is said to have been "*inactivated*."

Antimicrobic sera are not as efficient in treatment as antitoxic ones. It might be that if we could use homologous sera for treating man instead of the usual heterologous ones from the horse better results might obtain.

It would appear that a more hopeful outlook will obtain by combining serum therapy with chemotherapy, thus a combination of antipneumococcic serum with sodium oleate seems capable of producing curative results which neither alone can bring about.

Again, a combination of vaccination (active immunization) with the injection of the antimicrobic serum (passive immunization) has been thought by some to be of value.

Sensitization.—When we allow a mixture of bacteria or cells to remain in contact with their specific immune serum which has been inactivated, the amboceptors attach themselves to the bacteria or cells, so that now, upon adding normal serum (complement), these bacteria or cells are so prepared or mordanted that the complement can distintegrate them. This experiment of attaching amboceptors to cells is termed sensitizing and cells so treated are said to be *sensitized*.

Methods for Obtaining Immune Sera

While a convalescent from a disease may be utilized to obtain an antitoxic, agglutinating, opsonic, or bacteriolytic serum against the specific bacterium, yet this is more conveniently obtained from an animal which has been immunized against the bacterium or cell in question.

The rabbit is the most convenient animal to employ for the production of immune sera where the object is to have at hand a serum for use in diagnosis. Where sera are used on an extensive scale, as in the production of curative sera, large animals are employed.

Serum Diagnosis.—There are two applications of serum diagnosis:

1. Where the organism is known and the serum is to be diagnosed.
2. Where the serum is known and the organism is to be determined.

The first is employed by testing the agglutinating or bacteriolytic power of the serum taken from a patient upon pure cultures of the organism which is suspected as the cause of the disease. The Widal test (agglutination) is the best instance of this procedure. This method is of practical value in the diagnosis only of typhoid fever, undulant fever, and the paratyphoid fevers. In diseases like cholera and bacillary dysentery, the disease has run its course before agglutinating power becomes apparent in the serum. This method, however, may be used to prove that a convalescent has suffered from a suspected disease. Thus, by testing the agglutinating power of a serum, one or two weeks after recovery from a suspicious case of ptomaine poisoning, we may be able to demonstrate that the case in question was cholera. The second method has wider application, and is the one in which we use the sera of animals which have been immunized with known bacteria. Organisms isolated from urine, faeces, or blood of patients, or those obtained from water or food supplies, may be identified by testing the agglutinating, precipitating, opsonic, or bacteriolytic power of known sera against them. This has a wide range of applicability. The testing of the opsonic power of the sera in man or animals immunized against plague, and possibly cerebrospinal meningitis, seems to give more definite information than do agglutination or bacteriolytic tests. With the majority of other organisms, however, the agglutination test is the one almost always preferred.

Even in a small laboratory there are no particular difficulties in the way of having on hand rabbits immunized against typhoid, paratyphoid, undulant fever, acid-producing and nonacid-producing strains of dysentery, cholera, etc. Just as we inject men with vaccines prepared from various bacteria in therapy controlled by determinations of the opsonic index, so we inject animals to produce sera for diagnosis. We may use either a bouillon culture or the growth on agar slants taken up with salt solution as the inoculating material. This is heated for one hour at 60°C. to kill the bacteria. We have been injecting our animals with standard emulsions of organisms killed by addition of 0.1% formalin and kept in the ice box for 3 days. This does not affect the antigenic power of the bacteria. Where we desire to produce a serum which will disintegrate red blood cells (haemolytic serum), we inject intravenously about 1 cc., or intraperitoneally about 5 cc., of the washed red cells of the animal for which we wish to produce a specific serum. For details, see method of preparing haemolytic amboceptor serum under Noguchi's modification of Wassermann test.

Precipitating Sera.—For preparing a serum for the biological blood test we inject a rabbit intravenously with human serum in quantities of about 5 cc. every fifth day. About one week after the last injection the antiserum obtained from the injected rabbit should be strong enough for 0.1 cc. to produce turbidity when added to 1 cc. of a 1–1000 dilution of human serum in salt solution. Various controls are necessary when the test is used in medico-legal work.

Agglutinating Sera.—For obtaining an agglutinating or bacteriolytic serum for bacteria we inject about 1 cc. of the killed bacterial bouillon culture subcutaneously or into the peritoneal cavity of the rabbit. The easiest way to inject the rabbit is to

hold the animal head down and plunge the needle in the median line into the abdominal cavity, then forcing in the contents of the syringe. The intestines gravitate downward and by entering the needle below the limits of the bladder we avoid injuring any vital part. It may be more satisfactory to inject at first only about 0.5 cc., and then if there is very little reaction, as shown by the appetite and spirits of the

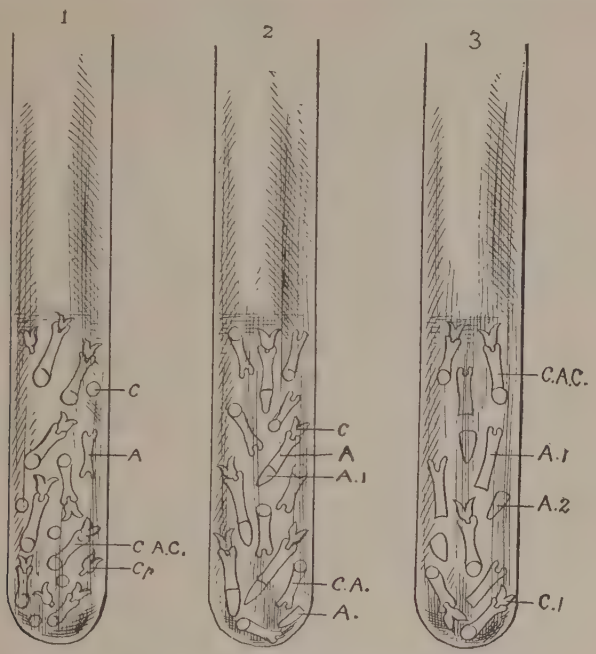


FIG. 60.—Mechanism of complement fixation.

Tube 1 shows the haemolytic system; *C*, a red blood corpuscle; *A*, a haemolytic amboceptor; *Cp*, complement; *C.A.C.*, complement united to a corpuscle by means of the specific amboceptor. Haemolysis results.

Tube 2 shows complement fixation by bacterial antigen and amboceptor; A_1, antigen; *C*, complement united to the antigen A_1 by the amboceptor *A*. When haemolytic amboceptors are added haemolysis does not occur because the complement has been previously fixed by the bacterial antigen and amboceptor.

Tube 3 shows absence of complement fixation because the bacterial amboceptor A_1 is not specific for the bacterial antigen A_2 and hence complement is not fixed; when haemolytic amboceptor and the corresponding corpuscles are added complement unites with these, *C.A.C.* and haemolysis occurs.

rabbit, to inject about four days later 1 cc. About four or five injections at intervals of three to five days will usually produce an immune serum.

Injection of the antigenic material (blood cells, serum or bacterial emulsion) into the marginal ear vein may be employed. With this method, however, I have had several rabbits die in what was considered anaphylactic shock. (For the method of

immunizing rabbits to produce a haemolytic serum see Wassermann test.) In immunizing rabbits for the production of monovalent sera for type agglutination of meningococcus and pneumococcus we inject $\frac{1}{100}$ of the surface growth of a slant the first day, $\frac{1}{75}$ the second day and $\frac{1}{50}$ the third day. Allow a week to pass and then start in again with $\frac{1}{50}$ the first day, $\frac{1}{25}$ the second day and $\frac{1}{10}$ the third day. If the titre is not high enough one can give $\frac{1}{5}$ of a surface growth on the fourth day. These injections are of living organisms. The English inject killed cultures. Some animals do not seem to be capable of producing antibodies, so that it may be necessary to use one or more rabbits before a satisfactory serum is obtained. The most convenient way of obtaining serum for a test is to cut across one of the marginal veins of the rabbit's ear, and collect the blood in a Wright's U-tube. After centrifugalization, the serum is ready for use.

The vein can be made to stand out prominently by applying a compress dipped into very hot water. When a large amount of serum is desired it is better to use a test tube with two pieces of glass tubing passing through a double perforated rubber stopper. To one of the projecting pieces of glass tubing a stout hypodermic needle is attached through the medium of 8 inches of rubber tubing and to the second piece of glass tubing passing through the stopper of the large test tube another piece of rubber tubing is attached for suction. To obtain blood from the rabbit find the ensiform cartilage and insert the needle in the notch to the left and gently force it upward. Applying suction with the mouth the blood flows into the test tube as soon as the needle enters the heart. By placing the tube of blood in the refrigerator the serum separates out from the clot. The removal of 20 to 30 cc. of blood does not seem to affect the animals in the least and they can be used in this way time and time again. The immune body and agglutinin in serum remain active for weeks when kept in the refrigerator. Such sera may also be dried on paper as for amboceptor paper (Noguchi). The complement and opsonin, however, begin to deteriorate at once and will have disappeared by the fifth day. Consequently, for opsonic and bacteriolytic and haemolytic experiments, fresh serum—twelve to twenty-four hours—must be used, or it may be activated.

Agglutinins

While Gruber and Durham first noted that cultures of *B. coli* and *Sp. cholerae* would develop flake-like clumps, when treated with the specific serum, it remained for Widal to apply this agglutination reaction to the diagnosis of typhoid fever. The agglutinating power of a serum is due to the presence of *agglutinin*, a specific antibody with both haptophore and agglutinophore receptors, which is elaborated by the lymphoid or possibly other body tissues in response to the action of a substance, agglutinogen, given off by bacterial or other alien cells introduced naturally or artificially. Agglutinins are more resistant than complement, withstanding a temperature of 60°C. for thirty minutes, while complement is destroyed at 55°C. Therefore in the inactivation of serum we do not destroy agglutinins.

If the temperature is raised above 60°C. the agglutinophore molecule is destroyed but the haptophore group is intact and such a damaged agglutinin can combine with its specific bacterium without, however, causing agglutination. To this we apply the name *agglutinoid*.

Usually a serum shows stronger agglutination when concentrated than when more diluted. We do observe reactions, however, in which the lower dilutions fail to agglutinate while the higher ones give agglutination. This is supposed to be caused by the presence of agglutinoids which have stronger combining power than agglutinins. In such cases we call these agglutinoids *pro-agglutinoids* and state that the paradoxical reaction is caused by the presence of pro-agglutinoids which must be absorbed before the agglutinins can cause clumping. The designation for the concentration in which agglutination fails to occur is *pro-agglutinoid zone*.

An immune serum will often agglutinate organisms closely related to its specific organism in higher dilutions than would a normal serum. Such agglutinins are called *group agglutinins* and their power is always markedly lower than the specific agglutinins.

It has been found that a serum treated with emulsions of its specific organism loses all its agglutinating power for that organism, and, along with this, for closely allied bacteria which it was previously capable of agglutinating in low dilutions. If, however, this serum is treated with one of the allied bacteria, instead of the specific one, the agglutinating power for the specific organism is but little affected. These reactions are spoken of as *agglutinin absorption* methods and are of value as showing whether an infection is a mixed one or only due to a single one of a number of organisms related from the standpoint of group agglutination. The agglutination for the specific organism or organisms is always much higher than for group-agglutinin organisms. Again, if an unknown organism removes all agglutinins from an immune serum we know that it is the same organism as the specific one, but if it only removes group agglutinins, leaving the specific agglutinins unbound, it is proven to be only a group organism. This method is probably the most exact one for determining the relationship of bacteria.

This is the best method for determining the different groups or strains of the meningococcus. At present we regard members of these meningococcus groups as being the same organism, although further study may show them to occupy the same relationship to one another as do the typhoid and paratyphoid organisms.

There are two methods of testing the agglutinating power of a serum—the microscopical and the macroscopical or sedimentation method. The phenomenon of agglutination as observed in typhoid and allied diseases is termed the Widal reaction.

1. **Microscopic Agglutination.**—For the microscopical method draw up serum to the mark 0.5 of the white pipette. Then draw up salt solution to the mark 11. This when mixed gives a dilution of 1 to 20. It is, however, more convenient to make the

serum dilutions with a graduated rubber bulb capillary pipette. Mixing one loopful of the diluted serum and one loopful of a bouillon culture or salt solution suspension of the organism to be tested gives a dilution of 1 to 40. Adding one loopful of the 1 to 20 diluted serum to 3 loopfuls of the bacterial suspension gives a dilution of 1 to 80. These two dilutions answer in ordinary diagnostic tests. The red pipette with a 1 to 100 to 200 dilution may be used where dilutions approaching 1 to 1000 are desired. Having mixed the diluted serum and the bacterial suspension on a cover glass, we invert it over a vaselined concave slide and examine with a high-power, dry objective ($\frac{1}{6}$ inch). It is neater to press down the vaselined periphery of the concavity on the cover glass. This sticks to the borders of the cover glass and the preparation is easily handled. It is simpler to make a ring of vaseline on a plane slide to fit the cover glass and make the mixture of diluted serum and culture in the center of this ring or square. Then apply the cover glass, press it down on the vaseline ring and examine as with the ordinary hanging drop. In making dilutions it is preferable to use salt solution, as the *phenomenon of agglutination requires the presence of salts.* Ordinarily, thirty minutes is a sufficient time to wait before reporting the absence of agglutination. Agglutination is more rapid at body temperature than at room temperature. In reporting agglutination, always give time and dilution. It is absolutely necessary that a control preparation be prepared in every instance; that is, one with the bacterial culture alone or with a normal serum of the same dilution as the lowest used. Some normal sera will agglutinate in 1 to 10 dilution, and *group agglutinations* (as paratyphoid with typhoid serum) may occur in 1 to 40 or possibly higher. It is very unusual for sera to agglutinate any other bacterium than the specific one in dilutions as high as 1 to 80.

2. Macroscopic Agglutination.—For the macroscopical or sedimentation test, take a series of small test tubes ($\frac{3}{8} \times 3$ inches) and deposit 1 cc. of salt solution in each of the series. Now, having taken an empty test tube, drop 4 drops of serum in it and then add 12 drops of salt solution. This gives 1 cc. of approximately a 1 to 4 dilution of the serum. It is more exact to make the 1 to 4 dilution with a graduated pipette. With a rubber-bulb capillary pipette, which has been graduated to hold 16 drops or 1 cc., draw up the contents of the tube containing the 1 to 4 serum and add it to the next tube containing 1 cc. of salt solution. This gives 2 cc. of a dilution of 1 to 8. Now mix thoroughly by drawing up and forcing out with the bulb pipette, and then withdraw 1 cc. and add to the next tube containing 1 cc. of salt solution. This gives a dilution of 1 to 16. Having mixed as before, again withdraw 1 cc. of the mixture and add it to the 1 cc. in the next tube. We now have a dilution of 1 to 32. Again withdrawing 1 cc. and adding it to the fourth tube containing 1 cc. of salt solution we have a dilution of 1 to 64. In tube 1 there is now 1 cc. of a dilution of the serum of 1 to 8; in tube 2, there is 1 cc. of a dilution of 1 to 16; in tube 3, of 1 to 32. Tube 4 contains 2 cc. of 1 to 64. The dilutions can be carried on in the same manner to any extent that may be desirable. In cholera agglutinations we may run up to 1 to 5000 or thereabouts. Of course, where such dilutions are employed, we generally start with 2 cc. of 1 to 50 in the first tube. When we have completed the series, each tube having 1 cc. of diluted serum, and the last 2 cc., we remove with the pipette 1 cc. from the last tube and discard it by ejection from the pipette leaving 1 cc. in the last tube. Now adding 1 cc. of a culture of typhoid or any other organism, we have the dilution of the serum in each tube doubled. Tube 1 now contains a serum in dilution

of 1 to 16, acting on the bacteria; tube 2 of a 1 to 32; tube 3 of a 1 to 64. Now place these tubes in the incubator and, after two to five hours or overnight, we examine for the clearing up of the bacterial suspension. If the serum in a certain dilution agglutinates, the clumps gravitate to the bottom and the upper part becomes clear. If so desired, these dilutions may be carried on to 1 to several hundred in the same way. It is safer to work with dead cultures instead of living ones.

Killed cultures.—To prepare, take a twenty-four-hour agar slant culture of typhoid or paratyphoid and emulsify in salt solution (about 6 cc. to a slant). By adding 0.1% of formalin to the typhoid emulsion and placing in the ice box the cultures will be found sterile in about three days. The emulsion should be shaken twice daily while undergoing sterilization in the ice box. Such cultures are not easily contaminated and appear to retain their agglutinable qualities for several months. The macroscopic methods are preferable with such dead cultures.

Combination of microscopical and macroscopical methods.—Microscopic: Prepare dilutions of serum as above described and take from each or several of the series a loopful of the diluted serum. For control use a loopful of salt solution. Place on a cover glass and add loopful of bouillon culture of the living organisms. Make hanging drop preparation, report after one hour at room temperature. Use $\frac{2}{3}$-inch lens for examination.

Macroscopic: Add to each tube of the series, including the control, an equal amount of an emulsion of killed organisms.

A very convenient method in general use in Germany is the following: Make dilutions of serum in ordinary test tubes ($\frac{3}{4} \times 6$ inches) as described for the small test tubes.

Then take a loopful (2 mg.) of growth from an eighteen to twenty-four hour old agar culture and emulsify it thoroughly in the dilution in the first test tube —repeat the process in the second tube and so on. This procedure is much safer than when live cultures are added with a pipette. Again the dilution is unchanged by this addition whereas it is doubled when an equal volume of culture is added to the diluted serum. A control should always be made in normal salt solution. After incubating, look for precipitates by tilting the fluid in the tubes to form a thin layer and to obtain the most advantageous light. A fine curdy, flocculent precipitate indicates agglutination, and a uniformly turbid emulsion a negative reaction.

The method of using a slide with two vaselined rings, one containing an emulsion in the specific serum and the other in salt solution, is of great practical value. This method is described under cholera.

Oxford University Standard Agglutination Methods (Dreyer).—In order to have a standard method for comparison of agglutination results following vaccination with typhoid or paratyphoid vaccines, as well as for the diagnosis of a typhoid-like infection arising in such a vaccinated person the Dreyer method is generally recommended. At present, however, there is doubt as to its clinical value. It cannot take the place of blood cultures.

The Dreyer method makes use of standardized emulsions of typhoid and the paratyphoids. They are prepared as follows: *B. typhosus* is subcultured daily in broth, for about ten days, to increase its agglutinability and reduce its auto-agglutinability. Finally it is planted in broth in partly filled flasks and incubated twenty-four hours. At the end of that time, 0.1% formaldehyd solution (full strength) is added, it is placed in an ice box for four or five days, and it is shaken repeatedly. The then sterile culture is ready to be standardized for (1) opacity and (2) agglutinability.

As a matter of fact, instead of the elaborate technique recommended by Dreyer, one can obtain satisfactory clinical evidence by agglutinating the three organisms against the serum of the cases to be diagnosed. Any marked fluctuation or notable rise in the titre of the serum against one of the organisms, while the agglutinating power for the other two remains constant, is a valuable diagnostic point.

In noninoculated persons who have not had typhoid (or paratyphoid) fever, agglutination in a dilution of 1 in 25 justifies a strong suspicion of typhoid (or paratyphoid) infection. But the test must be applied again in the course of a few days to ascertain whether there is any change in the titre of agglutination. Marked agglutination in a dilution of 1 in 50 or more is, nearly always, diagnostic of active typhoid (or paratyphoid) infection.

A *noninoculated* "carrier" will normally show no important change in the titre of his serum on repeated examination at short intervals.

Inoculated persons if quite recently inoculated will usually show a high titre of specific agglutination. A rapid rise in titre sets in within two to four days of inoculation. This is followed by a fall at first rapid, but subsequently becoming very slow, so that a relatively high titre is maintained for a long period, even for years. During this period examinations made at intervals of a few days give practically identical readings.

It follows that in the case of inoculated persons the diagnosis of active typhoid (or paratyphoid) infection will require two or more successive examinations of the serum.

(*a*) If the individual is suffering from active *typhoid* infection his titre of typhoid agglutination will exhibit the usual rise and subsequent regular fall seen in noninoculated subjects, but starting from and returning toward the higher base line of inoculated persons.

(*b*) If the individual is suffering from active *paratyphoid* infection one of three things may occur as regards his *typical* agglutination titre, namely:

1. No appreciable change may occur in the titre of typhoid agglutination.
2. A relatively slight rise may occur, followed by a fall toward the former level.
3. A marked rise may occur synchronous with the rise in paratyphoid agglutination titre, and subsequently followed by the usual fall toward the former level.

Meanwhile the titre of *paratyphoid* agglutination runs the normal course of rapid rise to a maximum (usually exceeding the maximum typhoid titre) followed by a fall, at first rapid and then slower as already described for typhoid subjects, and falling *below* the persistent base line of typhoid agglutination of inoculated persons.

In the case of **mixed infections** whether in inoculated or noninoculated persons the agglutinin curves for the different infecting organisms are usually not synchronous, and they pursue their ordinary course independently of each other.

Agglutination of Meningococci.—For diagnosis of a colony on a plate made from a carrier, inoculate a slant of blood or serum agar from the colony, which will furnish abundant growth for the macroscopic dilutions to be made the next day. Prepare a 1 to 100 dilution of a polyvalent serum and emulsify in this serum enough of the growth to give a distinct turbidity. Some prefer a 1 to 200 dilution.

Another tube (9 × 1 cm.) containing 1 to 50 normal horse serum should be treated in the same manner, since certain meningococcus-like organisms agglutinate in the horse serum as well as the immune serum. These tubes should be incubated at 55°C. for sixteen hours or over night.

In testing for types, prepare dilutions of monovalent sera running from 1–50 to 1–800; and add to each an equal amount of formalinized culture, thus obtaining dilutions of 1–100 to 1–1600, in which it is not unusual to obtain agglutinations with the specific organism.

Houghton has modified the slide agglutination method of Krumwiede as follows:

Take a large loopful of 1 to 10 normal horse serum. Emulsify the colony from a plate in this serum. If agglutination occurs it is not a meningococcus. If agglutination does not occur he rubs in a small loopful of polyvalent serum (undiluted). The curdy clumping which begins to show itself within a minute or so is distinctly observed with the naked eye, or better with a magnifying lens, or the ⅔-inch objective. After the preparation dries do a Gram stain to confirm the identification of the clumped organisms.

By successively using small loopfuls of type sera, before adding the polyvalent, one can quickly obtain evidence as to type to be later verified by macroscopic agglutination. (Along this line one can do slide agglutinations for pneumococcus types.)

Agglutination of Pneumococci for Type Determination.—Take a specimen of the albuminous pneumonic sputum and do a Gram stain. From this the type III organism may be recognized by its large capsule. Inoculate a well washed bean-sized piece of sputum, emulsified in sterile saline, into the peritoneal cavity of a mouse. The mouse usually shows marked signs of illness in six to eight hours. Remove a small drop of the peritoneal exudate with a capillary pipette and examine to note abundance of pneumococci. If abundant, kill the mouse and wash out the peritoneal cavity with 4 to 5 cc. of saline. Collect these washings in a centrifuge tube and centrifuge at a low speed to throw down cells and fibrin.

The supernatant bacterial suspension is then removed and transferred to a second centrifuge tube and centrifuged at high speed. Remove the supernatant fluid and emulsify the sediment in sufficient saline to make a moderately heavy suspension (about equal to an eighteen-hour bouillon culture of pneumococcus). Mix 0.5 cc. of the emulsion with an equal quantity of the diagnostic sera and incubate for one hour at 37°C.

Pneumococcus suspension 0.5 cc.	Serum I (1:20) 0.5 cc.	Serum II (undiluted) 0.5 cc.	Serum II (1:20) 0.5 cc.	Serum III (1:5) 0.5 cc.
Type I	++	−	−	−
Type II	−	++	++	−
Subgroups IIa, b, x	−	+	−	−
Type III	−	−	−	++
Type IV	−	−	−	−

Note.—For agglutination of red cells see Blood Transfusion Tests.

Precipitin Reactions

In the diagnosis of bacterial infections the agglutinating tests are so much more satisfactory that precipitin tests are rarely applied, but, as will be noted below, such a test has been recommended for the diagnosis of cerebrospinal fever.

Bacterial Precipitin Tests.—In the technique of precipitin reactions for bacteria one filters two or three weeks-old bouillon cultures of a given organism through a Berkefeld filter, obtaining a filtrate (*precipitinogen*) which should not only be perfectly transparent, but also sterile as subsequent bacterial growth would give turbidity similar to a positive reaction. The *precipitin*-containing serum is prepared by injecting rabbits intravenously with bacterial filtrates as prepared above or with the bacteria themselves. The methods are similar to those for preparing agglutinating or haemolyzing sera. As with the agglutination phenomena so also do we have with precipitation ones the occurrence of group precipitins and precipitoids. Normal sera never seem to contain precipitins.

Test: To four tubes each containing 2 cc. of the bacterial filtrate are added increasing quantities of the serum to be tested; 0.05 cc. to the first tube, 0.1 cc. to the second and 0.5 cc. to the third, and 1 cc. to the fourth. Controls of positive and negative precipitating sera should also be prepared. The tubes are not shaken and the reaction should be allowed five or six hours at room temperature before final readings are made. When the serum is strongly precipitating the clouding of the clear fluid should take place in ten to twenty minutes.

Medico-legal Test for Blood.—In the *biological blood test* rabbits are immunized intravenously either with whole blood taken in citrated salt or with serum alone. For class work I used the blood of a chicken injected intravenously into a rabbit. An immune serum thus prepared contains haemolysins as well as precipitins. The haemolyzing effect of such a serum on the fowl's nucleated red cells shows well when examined in a hanging drop. The bleeding of the rabbit should be done after a period of fasting to avoid any opalescence of the serum.

Precipitating sera should be kept in the cold and may have 0.1% carbolic acid added as a preservative, or they may be preserved on paper strips.

The suspected blood stain should be extracted with normal salt solution and should be filtered until perfectly clear. An approximate strength of 1 to 1000 of the blood is desirable. This can be estimated as given under albumin in urine, with the U-shape tubing.

Test: Place 2 cc. of the 1 to 1000 extract of the stain to be examined in tube 1, 2 cc. of 1 to 5000 in tube 2, and 2 cc. of 1 to 10,000 in tube 3, adding to each tube 0.1 cc. of the precipitating serum.

In another tube put 2 cc. of a 1 to 1000 salt solution dilution of the serum of the animal from which the suspected blood is supposed to have come and add 0.1 cc. of precipitating serum.

Various other controls as with normal rabbit serum, etc., are necessary for medico-legal application.

The tubes should not be shaken and may be kept at room temperature or in the incubator. A positive reaction appears in two or three minutes as a clouding at the bottom of the tube which becomes a distinct precipitate in fifteen or twenty minutes. Readings should be made at the end of twenty minutes as reactions occurring subsequently have no significance.

Precipitin Test for Meningococci.—Vincent has recommended a precipitin test for epidemic cerebrospinal meningitis which has the advantages of being simple, of giving information sooner than cultures and of possessing particular value in those cases when meningococci cannot be found in the smears or in cultures from the cerebrospinal fluid. It is performed by adding 1 or 2 drops of antimeningococci serum to a tube of fresh cerebrospinal fluid which has been cleared by centrifugalization for ten to fifteen minutes. After adding the serum the tube is placed in the incubator at 52°C. for two to five hours together with a control tube. The formation of a precipitate (turbidity) shows a positive test.

Precipitin Tests for Pneumococci.—A very valuable method is to add 0.5 cc. of very clear urine from a case of pneumonia to an equal amount of each of the three types of diagnostic sera.

A positive precipitin reaction shows as a faint cloud or heavy flocculent precipitate. The reaction is present in about 65% of cases due to Type I, II or III.

The precipitating substances may appear in the urine as early as twelve hours after the initial chill.

Krumwiede takes an albuminous specimen of sputum in a small beaker (if the sputum is very mucoid the test is not satisfactory in our hands) and heats it in a water bath to coagulate the albumin. The coagulum is then rubbed up with about 1 cc. of saline in the water bath for five minutes. The saline, which has taken up the soluble pneumococcus-precipitating substances, is then centrifuged to clarify and 0.2 cc. of this extract is deposited on the surface of 0.2 cc. of each of the three-type sera. A cloud at the junction of the fluids (ring test) shows to which group the case belongs.

Avery's method for grouping the pneumococcus (artificial mouse).—Prepare a medium containing 18 parts of a meat infusion broth, 0.3 to 0.5 acid to phenolphthalein, sterilized without pressure; one part of a sterile 20% solution of dextrose, and one part of defibrinated rabbit blood. This should be tubed in 4-cc. quantities and not reheated.

Care should be taken to obtain a specimen of sputum from the deep air passages and to avoid mouth secretions. A portion the size of a bean should be selected and washed even more carefully than for mouse inoculation, passing it 3 or 4 times through sterile salt solution. The growth of the pneumococcus in this medium is not dependent upon virulence, as in the mouse, therefore greater care must be taken to avoid contamination by mouth types, by careful selection and washing of the sputum. The sputum is then ground in a sterile mortar, adding 0.5 to 1 cc. of broth drop by drop. This emulsion is then introduced directly into the medium.

After inoculation, the tubes are incubated for five hours at 37°C. A smear is then made, stained by Gram, and a blood agar plate is inoculated.

The tube is now centrifuged at low speed for two minutes, just enough to throw down the red cells but not enough to bring down the bacteria, and the supernatant fluid is transferred into a second tube.

There are two methods of proceeding from this point.

I. *The precipitin method.*—To the above supernatant fluid add 1 cc. of sterile bile and place the tube in water bath at 37°C. for twenty minutes. If not clear at the end of this time, centrifuge. The clear fluid is then used for a precipitin test, according to the Blake method as follows:

Tubes are set up in the following manner:

Tube 1.	0.5 cc. Serum I	(1 to 10)	plus 0.5 cc. culture.
Tube 2.	0.5 cc. Serum II	(undiluted)	plus 0.5 cc. culture.
Tube 3.	0.5 cc. Serum II	(1 to 10)	plus 0.5 cc. culture.
Tube 4.	0.5 cc. Serum III	(1 to 5)	plus 0.5 cc. culture.

An immediate specific precipitin reaction occurs in the tube containing homologous immune serum. Incubation is usually not necessary. A precipitate in tube 2 and not in tube 3 indicates Subgroup II.

II. *The agglutination method.*—If bile is not at hand, an agglutination test may be made directly on the fluid after removal of the red cells.

Oliver's method for typing pneumococci.—A rapid method of pneumococcus typing which apparently gives very good results has been described by Oliver. The sputum is first smeared and stained to determine the presence of pneumococci. One to 1.5 cc. sputum is then placed in a clean test tube containing a glass rod and 0.1 or 0.2 cc. normal saline is then added and the mixture stirred vigorously. Sufficient normal saline is now added to make the resulting mixture filterable. After about 0.5 to 0.8 cc. have been added, 3 to 5 drops of undiluted ox bile are added to the contents of the tube. The tube is then immediately placed in water bath at 45° to 48°C. for 10 to 20 minutes to permit the bile to dissolve the pneumococci. The fluid is now cleared by filtration through Eimer and Amend best white filter paper No. 15, or by centrifugalization, at first slowly and then up to 2000 revolutions per minute.

Three-tenths to 0.5 cc. of the clear fluid is pipetted into each of three tubes and a drop of undiluted serum of the three groups is added.

With a positive test, clouding occurs almost immediately on the addition of the specific antiserum. The reaction will be more marked if the tubes are immersed in water at 48°C. for 10 to 20 minutes.

If the tubes are now placed in the ice box for several hours, the positive tube will show sedimentation of the specific proteid, leaving the supernatant fluid clear.

Formol-gel Test.—This test is based on a precipitin reaction, but demonstrates also an alteration in the coagulability of the serum in certain diseases. In this test, a drop of clear serum from the patient is placed on a slide which is then inverted over a watch glass containing a few drops of liquor formaldehydi. In cases of kala-azar the serum will solidify, appearing as an opaque, stiff jelly which adheres to the slide, while other sera will remain fluid, running off the slide when it is tilted. The reaction, while employed chiefly in the diagnosis of kala-azar, appears not to be specific since it has been reported for syphilis and other diseases.

The Kahn Reaction in the Diagnosis of Syphilis

The importance of a reliable precipitation test for syphilis is generally recognized. Such a test requires but one basic reagent aside from serum; is comparatively simple in its performance and is free from many sources of error inherent in a complement fixation method.

The phenomenon of precipitation with syphilitic sera was observed soon after that of complement fixation and many attempts have been made to apply this phenomenon in the development of a precipitation test for syphilis. Michaelis (1907), Porges and Meyer (1908), Jacobstahl (1910), Bruck and Hidaka (1911), Hecht (1915), Meinike (1917), Sachs and Georgi (1918), Dryer and Ward (1921) and Vernes (1923), developed such tests. Although the last four gained some recognition, none received wide acceptance.

Kahn, in 1921, began to study the precipitation phenomenon with syphilitic sera and found the following governing principles to be essential for optimum results: 1. Concentration of the ingredients which enter into the test. 2. High instability of the antigen dilution. 3. Correct quantitative relation between serum and antigen. 4. Hastening of the union between serum and antigen reacting substances by agitation. By applying these principles and paying particular attention to the standardization of the antigen and technique, Kahn has developed a test which, aside from its apparent specificity and uniformity of results, possesses the practical advantage of being completed within a few minutes after mixing the ingredients.

The Kahn test comprises five procedures, three of which are with serum—(1) the routine test, (2) the quantitative procedure and (3)

the presumptive procedure. The remaining two are with spinal fluids—(4) the qualitative and (5) quantitative procedures. Originally, Kahn recommended the use of "special" antigen in some of these procedures. For greater simplicity, however, standard antigen now is used throughout.

Reagents Employed.—Three reagents are employed in the tests with serum: (1) standard antigen, (2) serum and (3) physiologic salt solution.

Preparation of standard antigen.—The antigen is cholesterinized alcoholic extract of powdered beef heart which has been previously extracted with ether.

Powdered beef heart is obtainable on the market (Digestive Ferments Co., Detroit). A large number of hearts enter into the preparation of each lot of this product and a higher degree of uniformity is obtained than is possible when the powder is prepared on a smaller scale. If, however, it is desired to prepare the powdered muscle in the laboratory, it is done as follows: About 400 Gm. of heart muscle are cut out from at least three beef hearts and passed four times through a meat grinder. The ground material is spread into a thin layer on a porcelain platter or glass plate and dried by means of one or two revolving fans. After six or eight hours, when the exposed surface is relatively dry, the material is turned over and drying continued over night. When the layer of beef heart is in the form of a dry plate, it is broken up into small pieces and drying continued until the material is brittle. It is now ground into powder form by means of a mortar.

The unit amount of powdered beef heart for antigen preparation is 25 Gms. and it is extracted in a 250 cc. Erlenmeyer flask. This will yield about 75 cc. antigen. If larger amounts are desired, as many 25 Gm. quantities are employed as needed.

For ether extraction, 25 Gm. of powdered beef heart are placed in a 250 cc. Erlenmyer flask and 100 cc. ether (anesthesia) added. The flask is shaken from time to time for an interval of ten minutes after which the ether is filtered off through qualitative filter paper. The filtration process is hastened by applying gentle pressure to the beef heart with a spatula. Filtration is completed when such pressure does not cause drops of ether to pass through the funnel.

The moist beef heart is now returned to the original 250 cc. extraction flask. This may be done by first transferring the beef heart from the filter paper to a sheet of white paper and breaking the material up with a spatula into pieces small enough for the mouth of the flask. 75 cc. of ether are now added and the mixture again extracted for a ten minute interval and filtered as above.

After the second filtration of ether, the beef heart is transferred to the flask for a third time and 75 cc. of ether again added. The mixture is extracted for a ten minute interval and again filtered as described above.

The moist beef heart is transferred for the fourth and last time to the flask and 75 cc. of ether added, the mixture extracted for ten minutes and filtered. After gentle pressure with a spatula does not cause drops of ether to pass through the funnel —the endpoint employed in each of the four ether filtrations—the beef heart is trans-

ferred to a sheet of white paper and dried either at room temperature or at 37°C. The drying usually requires from 10 to 15 minutes. When no ether odor is detectable, the beef heart is ready for extraction with alcohol.

An alcoholic extract of the beef heart powder after extraction with ether is prepared as follows: The dry powdered muscle is weighed and placed in the 250 cc. Erlenmeyer flask used for ether extraction—taking care that the flask is dry and free from ether odor. There will be approximately 23 Gm. remaining of the 25 Gm. used in the ether extraction. 5 cc. of 95% alcohol are added per Gm. of powder. The flask is shaken for 10 minutes and extraction allowed to continue at room temperature (21°C.), in the dark, for 3 days without shaking. At the end of this period, the mixture is shaken for 5 minutes and filtered. The filtrate is kept in the dark at room temperature as stock antigen solution.

Cholesterin is added to the alcoholic extract in the proportion of 6 mg. per cc. of extract. The cholesterin is dissolved by rotating the flask in a water bath at 37°C. When all the cholesterin has been dissolved, the antigen is filtered to remove impurities and allowed to stand one day before titration. The cholesterinized extract is stable and may be stored in the dark at room temperature indefinitely.

The ether and alcohol employed should be of high purity and the latter should be 95%. Corks covered with thin, pure tinfoil have been found most satisfactory as stoppers for flasks used in antigen preparation and storing. Rubber as well as cork stoppers give off soluble elements into the antigen which modify the final product.

Titration of antigen.—The aim of titrating antigen for this test is to find the minimum amount of physiologic salt solution to use with antigen which will result in an antigen salt solution precipitate that is soluble on further addition of salt solution.

One cc. cholesterinized antigen is added to each of 5 standard antigen dilution tubes (5.5 cm. length and 1.5 cm. diameter). To five similar tubes are added the following amounts of physiologic salt solution, respectively: 0.8, 0.9, 1.0, 1.1 and 1.2 cc. Each salt solution tube is emptied into a given antigen tube and, without waiting to drain the salt solution, the mixture is immediately poured back and forth 5 or 6 times to permit thorough mixing. Each of the 5 antigen dilutions will show the presence of a definite precipitate.

The antigen dilutions are allowed to stand for 30 minutes after which the solubility in salt solution of each of the precipitates in the dilutions is tested as follows: 0.05, 0.025, and 0.0125 cc. amounts, respectively, of each antigen dilution are pipetted with a 0.2 cc. pipette graduated in 0.001 cc. to the bottom of each of three tubes (7.5 cm. in length and 1 cm. diameter). 0.15 cc. quantities of physiologic salt solution are now added to each tube. The rack is shaken vigorously for 3 minutes after which salt solution is added to each tube—1 cc. to the 0.05 cc. antigen amounts and 0.5 cc. to the others—and observation made as to whether or not the original antigen dilution precipitate has gone back into solution. The antigen dilution tube containing the smallest amount of salt solution in proportion to antigen, having a precipitate which goes back into solution in salt solution, represents the endpoint of this titration and determines the proportion in which antigen is to be mixed with salt solution in the performance of the tests.

The accompanying table gives an outline of a typical antigen titration:

TYPICAL ANTIGEN TITRATION FOR TEST WITH SERUM

Antigen Dilution Series.....	1	2	3	4	5
Antigen + Salt Solution cc.	1 — 0.8	1 + 0.9	1 + 1.0	1 + 1.1	1 — 1.2
Result of Dilution	Heavy precipitate in each antigen dilution				
Scheme Used in Testing Solubility of Precipitate in Each Antigen Dilution	Tube No. 1 2 3 *Antigen Dilution cc. 0.05 0.025 0.0125 Salt Solution cc. 0.15 0.15 0.15 Tubes are shaken 3 minutes, 1 cc. salt salt solution added to tube 1 and 0.5 cc. to tubes 2 and 3. All are observed for precipitates.				
Solubility of Precipitate as Determined by Three-tube Test	Precipitate Not Soluble	Precipitate Not Soluble	Precipitate Soluble	Precipitate Soluble	Precipitate Soluble
Standard Antigen Dilution			Antigen + minimum amount of salt solution giving precipitate which dissolves in salt solution		

* The antigen dilution should be permitted to stand 30 minutes before using.

If the antigen titration indicates that 1 + 1.2 produces an insoluble antigen precipitate, the titration has to be extended with increasing amounts of salt solution (1.3, 1.4, 1.5, etc.) until the amount of saline is found in which the antigen precipitate completely dissolves. If the antigen titer is considerably above 1 + 1.1 it may be reduced to this figure by diluting the antigen with 95% alcohol (cholesterinized antigen with 0.6% cholesterinized alcohol). Thus, if antigen titer is 1 + 1.5, three 5 cc. amounts of cholesterinized antigen are diluted with cholesterinized alcohol, sufficient to render 15, 30 and 45% dilutions, respectively. Each of the 3 diluted antigens on retitration will be found to have titers less than 1 + 1.5. However, it is not necessary to retitrate these antigens with saline. Employing the standard antigen already in use as a control, these antigens may be tested directly with serums of different potency employing in each case a titer of 1 + 1.1 and choosing the antigen giving results similar to the standard.

The preparation of antigen for this test should be in the hands of highly trained serologists in central laboratories. Antigen which is ready for use can then be supplied to smaller laboratories. Furthermore, in standardizing antigen for the first time, workers should procure a sample of the standardized product (Michigan Department of Health, U. S. Naval Medical School, etc.) for a comparison control. In shipping antigen, glass sealed ampules are best employed.

Preparation of serum.—The serum is separated from blood clot by centrifugation. It must be free from cells and other particles. Previous to its use in the test, the serum is heated in a water bath at 56°C. for 30 minutes. When heated serum is kept overnight in the icebox, it is reheated for 10 minutes at 56°C. before using.

Preparation of normal saline.—The saline is an 0.85% sodium chloride (chemically pure) solution in distilled water. It should be filtered before using. Sterility is not required.

Routine Test with Serum.—The test employs three different proportions of serum and antigen dilutions: 3 + 1, 6 + 1, and 12 + 1.

Technique.—Standard antigen dilution is prepared by diluting standard antigen with normal saline in the proportion indicated by the antigen titration. One c.c. of antigen diluted with proper amount of normal saline is sufficient for about 18 tests. Two or more cc. of antigen may be diluted for a larger number of tests. The antigen is measured into a standard dilution tube and the amount of salt solution indicated by titration into a similar tube. The salt solution is poured into the antigen and, without waiting to drain the tube, the mixture is immediately poured back and forth 6 times to insure thorough mixing.

Antigen dilution is pipetted after it has stood at room temperature for 10 minutes. It is always pipetted to the bottom of the tubes. The 0.05 cc. amounts may be pipetted with a 1 cc. pipette and the 0.025 and 0.0125 cc. amounts with a 0.2 cc. pipette. The amounts may be indicated with a wax pencil. The antigen dilution should be mixed frequently during the pipetting period to assure a uniform mixture.

Serum is added to the tubes immediately after the antigen dilution is pipetted. To each of the 3 quantities of antigen dilution is added 0.15 cc. of each serum to be tested. When the serums have been added to 10 or less tubes, the ingredients are thoroughly mixed.

The rack of tubes is shaken for three minutes. When a shaking machine is employed, its speed should be close to 275 oscillations per minute. Hand shaking should approximate this speed. One may shake intermittently.

Salt solution is added to each tube to make the reading easier. One cc. is added to the tubes containing 0.05 cc. of antigen dilution and 0.5 cc. to the other tubes.

The controls include known positive and negative serum tests; an antigen dilution control which consists of the last set-up of 3 regular antigen amounts with 0.15 cc. salt solution, and serum controls to establish their freedom from foreign particles. In the last case, each serum which gives a positive reaction is diluted with salt solution (0.1 cc. serum +0.3 cc. salt solution), thoroughly shaken and examined to make sure that it is free from cells and particles. If present, the serum is recentrifuged and the test repeated.

The tests are read in front of a partly shaded window having preferably north or south light to avoid direct sun rays. There should be no strong light—artificial or natural—striking over the reader's shoulders. If room in which tests are read has windows back of the reader, the shades should be lowered so as to dim the light. The negative serums appear opalescent and are readily distinguishable without lifting the tubes from the racks. The strongly positive serums show precipitates which are also easily read directly. Only the tubes showing weak reactions need to be

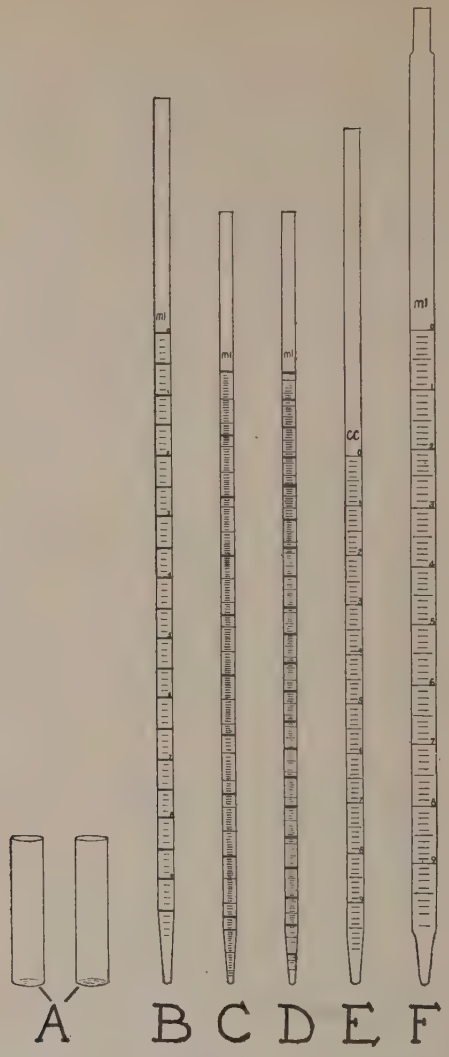


FIG. 61.—Antigen dilution tubes and pipettes.

A, Antigen dilution tubes employed in the preparation of standard antigen dilution. Inner diameter 15 mm. and length about 55 mm. *B*, Pipette, 1 cc. graduated in 0.01 cc. cap. 0.05 cc. graduations are indicated with a wax pencil. *C*, Pipette, 0.2 cc. graduated in 0.001 cc. cap. 0.025 cc. graduations are indicated with a wax pencil. *D*, Pipette, 0.2 cc. graduated in 0.001 cc. cap. 0.0125 cc. graduations are indicated with a wax pencil. *E*, Pipette, 1 cc. graduated in 0.01 cc. cap. Used for measuring serum. *F*, Pipette, 10 cc. graduated in 0.1 cc. cap. Used for measuring saline for final dilution of tests.

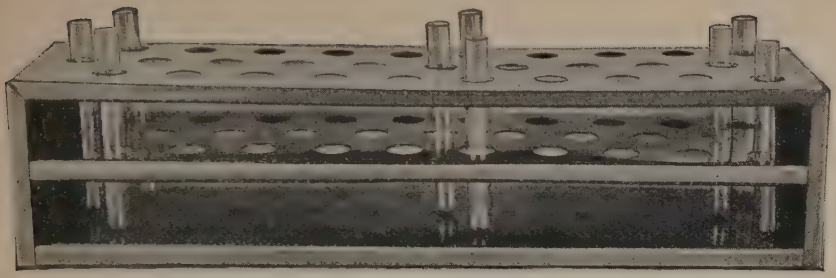


FIG. 62.—Standard rack and test tubes.

Made of sheet copper, 3 inches wide by 11.5 inches long by $2\frac{3}{4}$ inches high. Consists of three shelves, upper and middle ones containing 3 rows of 10 holes, each of approximately $\frac{1}{2}$ inch in diameter. The center row holes offset $\frac{1}{2}$ inch. The bottom shelf serves as support.

Test tubes in carrying out precipitation tests. Inner diameter about 10 mm. and length about 75 mm.

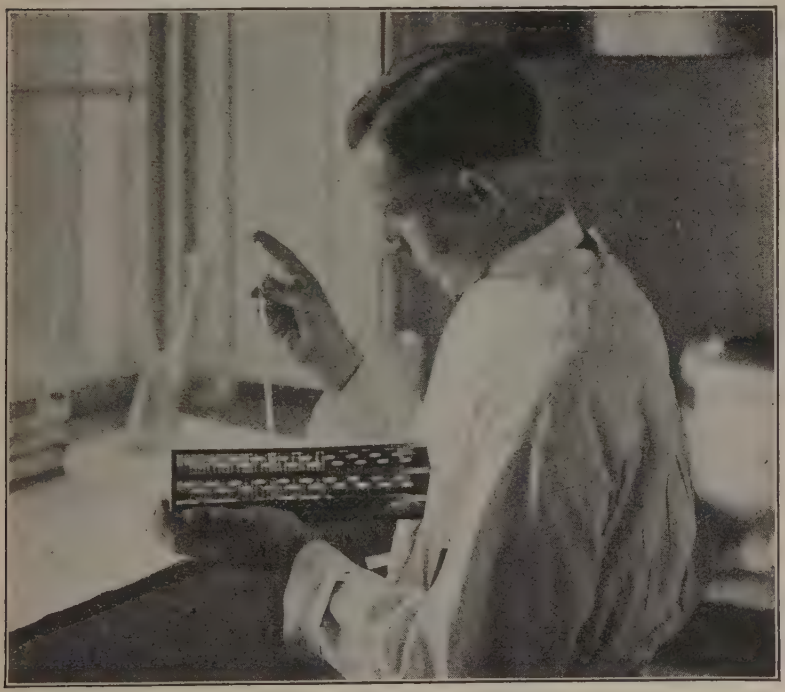


FIG. 63.—Pipetting antigen dilution.

In pipetting the small amounts of antigen dilution it is necessary to deliver to the bottom of the tube.

FIG. 64.—Pipetting of serum.

In pipetting these amounts it is not necessary to lower the pipette to the bottom of the tube.

FIG. 65.—Shaking.

During the shaking period it is important not merely to agitate the rack and tubes, but to see that the fluids within the tubes are vigorously shaken.

removed from the rack and examined individually. Each tube is lifted from the rack for some inches above the eye level, slanted to spread the fluid into a thin layer and examined for a precipitate.

When it is desired to read the Kahn test by artificial light, the following slit-light arrangement will be found to give good results. A wooden or metal box about 8 inches high and 4 inches square is so constructed as to permit an electric bulb attachment at its inner base and an $\frac{1}{8}$th to $\frac{1}{16}$th inch slit at its top. On lighting the electric bulb and holding the tube in a slanted position over the slit of light, positive sera

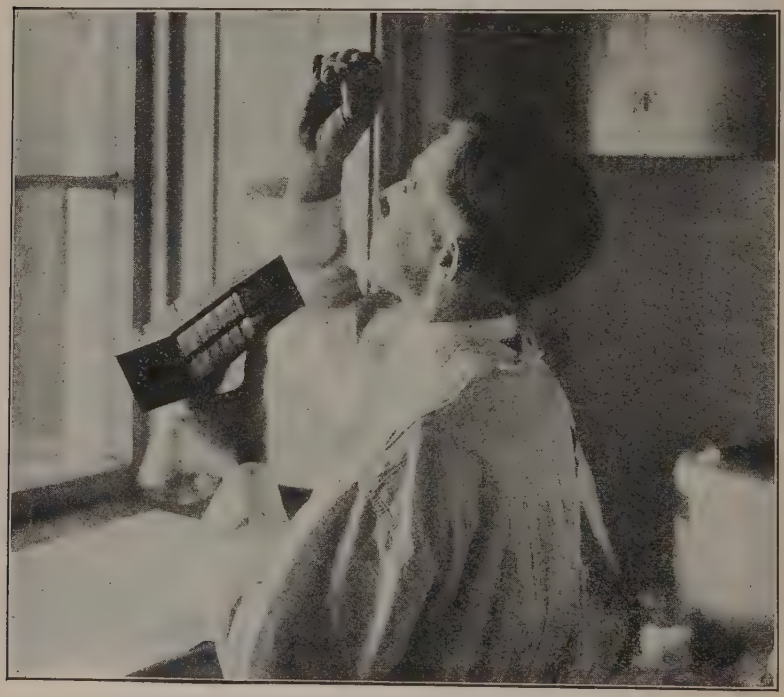


FIG. 66.—Reading results.

Readings are made in front of a partly shaded window with a darkened background. The strongly positive sera show heavy precipitates which are readily distinguishable without lifting the tubes from the rack. In the case of the weakly positive sera, each tube is examined as illustrated.

will show definite precipitates while negative sera will be free from precipitates. A 25 watt frosted bulb will give good results.

A definite precipitate suspended in a clear medium is considered a completed reaction and is read ++++. Proportionally weaker reactions are read +++, ++, and +, respectively. The final result in each test is the average finding of the three tubes. Thus, if the precipitation reaction is ++++ in each of the three tubes,

the final result is ++++. If the reaction is −, ++, ++++, the final result is ++. A number of typical reactions with this method and the final result in each case, are illustrated in the accompanying table.

Outline of Kahn Test and Interpretation of Results

THE TEST

Tube No.	1	2	3	Completion of test
Serum + Antigen dilution..	3 + 1	6 + 1	12 + 1	Tests shaken three minutes, 1 cc. salt solution is added to first tube and 0.5 cc. to other two tubes and results read.
Antigen dilution, cc.....	0.05	0.025	0.0125	
Serum (heated at 56°C. for 30 min.) cc.....	0.15	0.15	0.15	

INTERPRETATION OF RESULTS

	Reaction No.				Final result (average of reactions of three tubes)
Some typical reactions	1	++++*	++++	++++	++++
	2	+++	++++	++++	
	3	++	++++	++++	+++
	4	+	+++	++++	
	5	−	+++	++++	++
	6	−	++	++++	
	7	−	+−	++++	+
	8	−	−	+++	
	9	−	+−	++	+−
	10	−	−	−	−

* The precipitate in the first tube is frequently heaviest in ++++ reactions due to the large amount of antigen dilution it contains and lightest in the third tube due to the small amount of antigen dilution.

Quantitative Procedure.—This procedure determines the relative number of reacting substances (Kahn units) in positive serum. The method consists in testing a series of serum dilutions with a constant amount of standard antigen dilution. If a serum gives a definite precipitate only when undiluted it is considered as containing 4 reacting units. If a serum gives a definite precipitate in a dilution of 1:5, it contains 20 reacting units. Similarly, the potency of any serum can be determined according to the formula S = 4D, where S = potency of serum in terms of reacting units and D = highest dilution giving a definite precipitation reaction.

Technique.—A series of serum dilutions ranging from 1:1 to 1:60 with saline are prepared as follows:

1:1 = undiluted serum.
1:5 = 0.2 cc. undiluted serum + 0.8 cc. salt solution.
1:10 = 0.7 cc. 1:5 dilution + 0.7 cc. salt solution.
1:20 = 0.2 cc. 1:10 dilution + 0.2 cc. salt solution.
1:30 = 0.2 cc. 1:10 dilution + 0.4 cc. salt solution.
1:40 = 0.1 cc. 1:10 dilution + 0.3 cc. salt solution.
1:50 = 0.1 cc. 1:10 dilution + 0.4 cc. salt solution.
1:60 = 0.1 cc. 1:10 dilution + 0.5 cc. salt solution.

(If it is desired to make more exact determinations of the number of reacting units in certain cases, serum dilutions in between those already indicated may be included. Serums of high potency, which give complete precipitation in a 1:60 dilution are diluted still further until a negative reaction is obtained.)

The antigen dilution is prepared as for the routine test and after 10 minutes standing is pipetted in 0.01 cc. quantities to the bottom of each of eight tubes. This is followed by 0.15 cc. amounts of the various serum dilutions, in order, beginning with undiluted serum. The rack is shaken for 3 minutes, 0.5 cc. salt solution added to each tube, and the results read.

Interpretation of results.—Tubes showing definite precipitates, such as ++++, +++ or ++ reactions are read positive. Those showing + or negative reactions are read negative; and the number of reacting units in a given serum is 4 times the maximum dilution of the serum showing a definite precipitate.

Presumptive Procedure.—This is a simple one tube procedure slightly more sensitive than the routine test and intended largely as a check on this test.

Technique.—Standard antigen is diluted according to the titer and allowed to stand 10 minutes before use. For each specimen to be tested 0.01 cc. antigen dilution is pipetted to the bottom of a standard test tube and 0.15 cc. patient's serum added and thoroughly mixed. The mixture is shaken for 3 minutes vigor-

ously, 0.05 cc. saline added and the results read. As in the quantitative procedure, a definite precipitation reaction (++++, +++ or ++) is read positive; all others are read negative. The controls are similar to those employed in the routine test.

Spinal Fluid Procedure.—The test with spinal fluid (qualitative and quantitative) involves a preliminary concentration of the syphilitic reacting substances by precipitation of the globulins and their solution in an amount of salt solution equal to one-tenth the original volume of spinal fluid.

Preparation of globulin solution.—The spinal fluid is first centrifuged to render it entirely free from cells and foreign particles. Three cc. of the clear fluid are placed in a 10 or 15 cc. conical shaped centrifuge tube and 2 cc. saturated ammonium sulphate (Merck's reagent or Baker's Analyzed) added. After thorough mixing, it is placed in a water bath at 56°C. for 15 minutes to hasten precipitation of the globulins. It is then centrifuged at high speed for about 15 minutes to completely throw down the precipitated globulins. *The supernatant fluid is removed as completely as possible* by means of a capillary pipette having a rubber bulb. The globulin precipitate is then dissolved in 0.3 cc. saline, adding this after lowering the pipette close to the precipitate to avoid washing down ammonium sulphate from the inner wall of the tube. The dissolved globulin precipitate is now ready to be tested with antigen dilution. (If only 1.5 cc. spinal fluid is available, add 1 cc. saturated ammonium sulphate and dissolve globulin precipitate in 0.15 cc. saline.)

Standard antigen dilution.—To counterbalance the presence of some ammonium sulphate in the globulin solution, it is found that the titer of the antigen is 0.4 cc. higher than for the routine test. Thus, if the titer with serum is 1 + 1.1, the titer with spinal fluids is 1 + 1.5.

Technique.—Antigen dilution is prepared according to required titer and allowed to stand 10 minutes. Two 0.01 cc. quantities of this dilution are pipetted into two small tubes; 0.15 cc. of the concentrated globulin solution added to each and thoroughly mixed. The mixtures are shaken vigorously for 3 minutes, 0.5 cc. salt solution added to each tube and the results read. A definite precipitate suspended in a clear medium is read ++++. Proportionally weaker precipitates are read +++, ++ and + respectively.

Quantitative Spinal Fluid Procedure.—This procedure is applicable to fluids giving strongly positive reactions with the qualitative test. A standard amount of antigen dilution is used with various dilutions of the concentrated globulin solution and of the spinal fluid. The final results are interpreted in terms of reacting (Kahn) units.

Technique.—When 3 cc. of spinal fluid is employed, the final globulin solution—0.3 cc.—is sufficient for both the qualitative and quantitative tests. If the qualitative test employing 0.15 cc. of globulin solution with 0.01 cc. antigen dilution, proves

to be positive, the quantitative procedure is carried out with the remaining 0.15 cc. globulin solution as well as with the original spinal fluid. Four spinal fluid dilutions with saline are prepared which are equivalent to 1:5, 1:10, 1:15, and 1:20 dilution of the globulin solution. To prepare the 1:5 dilution, 0.6 cc. saline are added to the 0.15 cc. globulin solution. For the 1:10 dilution, the original spinal fluid is employed. For the 1:15 dilution 0.2 cc. of the original fluid is mixed with 0.1 cc. saline. For the 1:20 dilution 0.2 cc. of original fluid is mixed with 0.2 cc. saline. Antigen dilution is mixed as for qualitative spinal fluid test and after 10 minutes standing is pipetted in 0.01 cc. amounts to four tubes. This is followed by 0.15 cc. amounts of the diluted fluid beginning with 1:5 and ending with 1:20 dilution. The tubes are shaken for three minutes; 0.5 cc. saline added to each tube and results read. A definite precipitation reaction (++++, +++ or ++) is read positive; all others are read negative.

Interpretation of results.—If only the concentrated fluid gives a definite precipitation reaction, the final result is four units. As is true in the quantitative serum test, the number of reacting units in a spinal fluid is 4 times the maximum dilution of the concentrated fluid showing a definite precipitate. Thus, if tube containing 1:5 dilution gives a definite precipitate, the final result is 5×4 or 20 units; if 1:20 dilution shows a definite precipitate, the final result is 80 units.

Micro Kahn Reactions.—The following plan is carried out when sufficient serum (0.45 c.c.) is not available for a complete three-tube Kahn test:

1. If 0.3 cc. is available, a two-tube test is made, employing the two lesser amounts of diluted antigen (0.025 and 0.0125 cc.). The results are averaged as though a regular three-tube test were made.

2. If 0.15 cc. serum is available, a one-tube test is made, using the smallest amount of diluted antigen (0.0125 cc.). In this case, definite precipitation reaction, such as ++++, +++ or ++, is read positive; all others are read negative. The results are reported "Positive (or negative) based on incomplete test; insufficient serum for complete (three-tube) Kahn test."

3. When less than 0.15 cc. serum is available, a test is made using a proportion of ten parts of serum to one part of diluted antigen. Thus, if only approximately one-half drop (0.025 cc.) of serum is available, the test is performed as follows: 0.0025 cc. diluted antigen is measured with a 0.1 or 0.2 cc. pipette graduated in 0.001 cc. and deposited at the bottom of a small tube of about 0.5 cm. diameter. With a similar pipette, 0.025 cc. serum is added close to the bottom of the tube. The rack is shaken three minutes, 0.2 cc. of physiologic sodium chloride solution is added, and the results are read. The report to physicians is made as in test 2, outlined above.

Similarly, Kahn tests can be carried out with 0.1 and 0.05 cc. amounts of serum, using 0.01 and 0.005 cc. of diluted antigen, respectively. Repetition of the test with physiologic sodium chloride solution instead of serum serves as the antigen control. Examination of the serum to establish freedom from cells and other particles serves as the serum control.

"Local" Kahn procedure.—After cleansing, and under moderate pressure, serum from chancres or other syphilitic lesions is collected by means of a fine capillary pipette. This is deposited at the bottom of a small agglutination tube and

centrifuged to throw down cellular matter. The supernatant serum is to be used in the test.

Method.—Standard antigen is prepared in the usual manner as for the routine test for blood serum, and 0.005 cc. of the antigen dilution is deposited by means of a 0.2 cc. pipette to the bottom of a small agglutination tube. This is followed with 0.05 cc. of the supernatant serum. The mixture is shaken three minutes, 0.3 cc. saline added and the results read. A definite precipitate is read positive while freedom from a precipitate is read negative.

If difficulty is encountered in collecting 0.05 cc. of serum from the syphilitic lesion, normal saline may be added to bring it to this amount before performing the test.

Technical performance of the various Kahn procedures should be in the hands of workers properly trained in clinical laboratory methods.

Clinical Application of the Different Kahn Procedures.—1. The routine test is a conservative method in the diagnosis and treatment of syphilis. It is somewhat more sensitive than the Wassermann test in treated cases and in early primary. The results are interpreted on the basis of plus signs. 2. The quantitative procedure is of especial value as a quantitative check on treatment. The results are interpreted on the basis of serum reacting (Kahn) units. 3. The presumptive procedure is somewhat more sensitive than the routine test and is employed as a check on this test. The results are interpreted as positive or negative. 4. The spinal fluid qualitative procedure is used as an aid in the diagnosis and treatment of neurosyphilis. The results are read on the basis of plus signs. 5. The spinal fluid quantitative procedure is of especial value as a check on treatment and the results are read on the basis of spinal fluid reacting (Kahn) units. "The correlation, by clinicians, of the results obtained with these different procedures in a large number of cases should ultimately lead to a better understanding of the interpretation of serologic reactions in syphilis."

General Considerations.—The Kahn test has the distinction of being reported upon favorably in the literature by practically all workers. For more recent reports one might mention the studies of Schueren, Owen and Cope, Redfield, Faupel, Kelley, Lederer, Dutton, Litterer, Duemling, Giordano, Hopkins and Brunet, Berry, Ey and Delong, Walker, and Houghton, Hunter and Cajigas. The Michigan Department of Health, after reporting 175,000 parallel Kahn and Wassermann tests to Michigan physicians during a period of three years, made the Kahn test the standard procedure beginning with October, 1925. By June, 1927 this Department reported over 125,000 Kahn reactions with satisfactory results. Parham and Behrens and particularly Houghton studied the Kahn test with highly satisfactory results in the U. S. Navy and in December, 1925 the Bureau of Medicine and Surgery adopted the Kahn test as the standard method in place of the Wassermann test. The outstanding features of the Kahn test are relative simplicity of technique, ready availability in laboratories throughout the world and comparative freedom from sources of error.

Deviation of the Complement

It has been found that if there is not sufficient inactivated immune serum added to a mixture of normal serum (containing abundant complement) and emulsion of living bacteria only a portion of the bacteria will be destroyed. Increasing the amount of immune body with a constant quantity of normal serum, we reach a point where all the bacteria are destroyed. Now, if we continue to increase beyond this point the addition of immune serum, the destruction of the bacteria ceases, and the cultures will contain an increasing number of living bacteria (*Neisser-Wechsberg Phenomenon*).

To carry out the test, make a series of tubes containing mixtures of bacteria with the same quantity in each of normal serum. Thus, each tube contains 0.5 cc. of bacterial emulsion and 0.5 cc. of 1 to 10 normal serum. Now inactivate a tube of 1 to 100 immune serum and add this to each of the tubes of normal serum and bacterial emulsion, increasing the amount with each successive tube. Thus, 1 drop to No. 1 tube, 2 drops to No. 2 tube, and so on. After incubating for two hours, we take a pipette and plate out a fraction of a drop in an agar plate. The limit at which bacteriolysis is complete is shown by failure of colonies to develop. Beyond or below that point colonies are more or less abundant.

The explanation of this phenomenon of deviation or deflection of the complement is that where we have an excess of amboceptors for available receptors on the bacterial cells, only a portion of the amboceptors can attach themselves to their specific bacteria. The free amboceptors, not being able to form a union with the bacterial cell receptors (for which they have a greater affinity), combine with the complement present. Unless the complement be in excess, there will be no free complement left to join on to the amboceptors attached to the bacterial cells, and consequently bacteriolysis does not take place and the plate cultures show an abundance of colonies. Zinsser regards the true explanation of this phenomenon as one similar to proagglutinoid ones where too concentrated serum dilutions fail to produce agglutination.

Stimson has found, in titrating his complement and amboceptor for complement fixation tests, that keeping his complement content constant and successively increasing the amount of amboceptor give increasing haemolyzing effect up to a certain point, beyond which the further addition of amboceptor causes a lessening of haemolytic power.

This he regards as due to deviation of complement and in his tests he prefers to keep a fixed amount of amboceptor and adjust his titrations by increasing complement rather than amboceptor.

Fixation or Absorption of the Complement

One of the controversies in connection with the nature of the complement is that regarding the question of the unity of complements or

whether there exist different kinds of complements for different amboceptors (unity and multiplicity of complement). To prove that a single complement will act with varying amboceptors, Bordet and Gengou showed that the same complement would unite with both haemolytic and bacteriolytic immune bodies.

If to a mixture of typhoid bacteria and inactivated typhoid immune serum some guinea pig serum is added and the mixture allowed to remain at 37°C. for two hours, and then sensitized red cells be added and the mixture again placed in the incubator for two hours, no haemolysis will be found to have occurred, because the bacteria have absorbed all the guinea pig complement through the intervening typhoid amboceptors, and there is no complement left to haemolyze the red cells through the specific red blood cell amboceptors.

If, instead of immune typhoid serum, the serum of a normal person had been used, there would have been no amboceptors to unite the complement to the bacterial cells. The complement would then be left free to unite with the sensitized red cells subsequently added and bring about their haemolysis, as shown by the ruby red color of the fluid.

Complement Fixation in the Diagnosis of Syphilis

Wassermann, Neisser and Bruck, in 1906, were the first to utilize the phenomenon of Bordet and Gengou in the serologic diagnosis of syphilis. At first the reaction, as applied to syphilis, was thought to be a true antigen-antibody reaction. It is now well recognized, however, that the body in a syphilitic serum which reacts with the antigen is not an amboceptor, but a lipoidophilic substance which has the property of linking complement to the lipoidal antigen. The name "reagin" has been proposed for this lipoidophilic substance. A similar substance is present in the serum of patients suffering with yaws and the Wassermann reaction is just as constant in that disease as in syphilis.

In the complement fixation tests most widely used to detect the presence of this reagin, there is employed either the antisheep haemolytic system (Wassermann test and its modifications) or the antihuman haemolytic system (Noguchi test and its modifications). Various other haemolytic systems such as the antiox, antichicken, etc., may also be used. Kolmer in his standardized technique recommends either the antisheep or the antiox haemolytic system.

Properly speaking, the term "Wassermann reaction" should be limited to the test conducted after the original method of Wassermann, but, practically, it has become synonymous for the complement fixation test as used for the diagnosis of syphilis.

The Antisheep Complement Fixation Test for Syphilis (*Wassermann Test*)

There have been numerous modifications and attempts at standardizing the Wassermann test, and many of these are undoubtedly of equal merit, but space will not permit a description of all the various methods. The following modification of the Wassermann test devised by Neill is the one now in use at the Hygienic Laboratory, U. S. Public Health Service and has given satisfactory results.

An excellent description with detailed specifications of the apparatus used in the complement fixation test for syphilis is given in reprint No. 483, U. S. Public Health Reports.

In handling the reagents and glassware chemical and bacteriological cleanliness should be observed. Glassware should be thoroughly rinsed with boiling water and allowed to dry without wiping. Ordinary soaps, weak solutions of acid and alkali, bichloride of mercury, and other chemicals have a destructive action on the delicate erythrocyte, and test tubes which have been in contact with these substances must be thoroughly washed and rinsed in clear running water, finally being boiled in pure water and dried previous to use in the test.

Preparation and Preservation of Reagents

Sodium chlorid solutions.—A nine-tenths per cent. solution of the chemically pure salt in distilled water is made up by weight, distributed in 500 cc. Erlenmeyer flasks and sterilized by steam.

Saturated sodium chlorid solution.—Is made by saturating a small bottle of sterile distilled water with the chemically pure salt. The bottle is to be kept at room temperature.

Sheep's blood corpuscles.—These are best obtained by bleeding a sheep from the jugular vein by use of a sterile syringe, previously rinsed with saline solution, transferring the blood immediately to a sterile 50 cc. Erlenmeyer flask containing sterile glass fragments, and agitating for 15 minutes, avoiding foaming, thus defibrinating the blood. Sheep's corpuscles can also be obtained at the abattoir by catching the blood coming from the vessels of the sheep's neck directly in the defibrinating flask. In doing this due care should be taken to avoid the entrance of gross dirt into the flask. After defibrinating, decant the blood into a graduated cylinder which is either perfectly dry or has just been rinsed out with 0.9 per cent. sodium chlorid solution. Note the amount of blood and divide it about equally between two or more 50-cc. centrifuge tubes. Add 0.9 per cent. sodium chlorid solution till tubes are nearly full, and mix thoroughly. The glass centrifuge tubes in place within the metal tubes and trunnion rings in place should be balanced against each other in pairs. Centrifuge till the corpuscles are completely precipitated. Pipette off supernatant fluid, add fresh 0.9 per cent. sodium chlorid solution, mix thoroughly, centrifuge again, pipette off the supernatant fluid, add fresh sodium chlorid solution, mix, and again centrifuge. After the third centrifuging, pipette off the fluid above the blood cells, transfer the cells to the cylinder previously used for measuring the

volume of the blood, which should be freshly rinsed with saline solution, and make up to the original volume with 0.9 per cent. sodium chlorid solution. Keep blood corpuscles in the ice box at a temperature not above 6°C. Cells may thus be preserved for at least 48 hours. The blood cells should not be used if the supernatant sodium chlorid solution shows a reddish coloration. If after the final washing a sterile 5 per cent. dextrose solution be used to make the corpuscle suspension up to the original volume of the blood, the corpuscles should show no evidence of disintegration at the end of 10 days, if kept at a temperature of not more than 6°C. Under these conditions they may be used in the test the same as fresh cells.

Hæmolytic amboceptor.—This substance is produced in the serum of a rabbit by injecting this animal with the washed blood corpuscles of a sheep prepared as above. The following methods of producing hæmolytic amboceptor may be used:

1. Inject rabbits intravenously with 1 cc. of fresh sheep cell suspension on the first, fourth, seventh, and tenth days of the process. Make test bleeding on the fifteenth day.

2. Inject rabbits intravenously with 1 cc., 1 cc., and 2 cc. washed sheep corpuscles on three successive days. Wait five days and repeat the injections. Make test bleeding five days after the last injection.

When the rabbit sera have been found to contain hæmolytic amboceptor of a suitable strength, the animals should be exsanguinated into large sterile centrifuge tubes or test tubes. When the blood has clotted, separate the clot from the sides of the tube by means of a platinum wire, or glass rod, and place the tubes in the 37°C. water bath for an hour, then place in the cold box over night. Separate the serum, add 0.3 per cent. phenol or an equal volume of high grade sterile neutral glycerin and preserve in the ice box at a temperature not higher than 6°C. Amboceptor serum should be stored two weeks before use.

Complement.—Bleed not less than five full-grown guinea pigs from the heart by means of a sterile syringe, previously rinsed with salt solution. With proper technique, from 5 to 10 cc. may be obtained without injury to the animal. Pigs may be kept for this purpose and bled once in two weeks. Guinea pig complement varies, being reduced for example when the pigs are on a diet too rich in greens. Place the blood drawn from each pig in a separate centrifuge tube, and allow to clot. Separate the clot from the side of the tubes with sterile needle or pipette and place in 37°C. water bath for one hour. Then place in cold box overnight, or, if serum has separated pipette off the serum immediately. Centrifuge clear of red blood corpuscles and pool the sera in a sterile glass container. For each cubic centimeter of pooled sera add one-tenth cubic centimeter saturated sodium chlorid solution, mix well, and preserve in the cold box at a temperature not above 6°C. Guinea-pig serum so preserved will retain its complement undiminished in hæmolytic properties for two weeks. Just before use dilute with three volumes of distilled water, to restore the normal tonicity, and dilute as convenient with 0.9 per cent. sodium chlorid solution.

Antigen.—A suspension of ether and alcohol-soluble, acetone-insoluble lipoids is used in the test. This is prepared as follows: A fresh beef heart is freed from fat and connective tissue and the muscle ground in a meat grinder. Further comminution is not essential, but a greater yield of antigen is secured by hand grinding in a mortar with sand. Place 200 Gm. of the ground meat in a bottle, add 2000 cc. of

alcohol (either absolute or 95%) and stopper tightly. Extract for at least two weeks at 37°C., thoroughly shaking the bottle three times daily. Filter through filter paper and evaporate with the aid of a vacuum and condenser, keeping the solution at somewhat below 40°C. by means of a water bath. Agitate the flask from time to time to keep the material washed down as it dries. When evaporated to dryness, wash out the residue with 100 cc. of ether, or as much more as may be necessary, and allow this to stand in a stoppered separatory funnel over night. Decant the clear, supernatant liquid and discard the residue. Evaporate the ethereal solution to secure a saturated solution which will reduce the volume to about 30 cc. Add at least ten volumes of acetone, stir, and allow to stand covered in a cool place over night. Collect the precipitate and bottle it with a little acetone. Preserve in the cold box.

For use, dissolve 0.3 Gm. of the solid in 1 cc. ether and 10 cc. of the best obtainable grade of methyl alcohol. Preserve this solution in the cold. When about to make a test, place the necessary amount of methyl alcohol solution in a dry vessel and add saline for dilution, the first 10 cc. slowly and the rest at convenience.

Cholesterinized antigen is prepared from plain alcoholic extract of beef heart—3 parts, and a saturated solution of cholesterin in absolute alcohol—2 parts. Keep solutions separated, the former in a cold box and the latter in a dark place at room temperature and mix just prior to using.

Note.—In the laboratory of the Naval Medical School the cholesterinized antigen is prepared by adding 0.1 to 0.2% cholesterin to the acetone-insoluble antigen or preferably to the stock antigen as prepared for the Kahn precipitation test. Alcoholic extracts of wet heart muscle are now little used, due to their anticomplementary properties and tendency to non-specific fixation.

The patient's serum.—Blood may be obtained from the arm vein. To do this sterilize a syringe and its needle by boiling, and also sterilize the skin of the front of the arm at the bend of the elbow. Rinse the syringe with saline solution. After the patient has opened and closed the fist vigorously several times, to pump the blood into the veins, place a tourniquet above the elbow just tightly enough to cut off the venous circulation. Puncture a prominent vein with the needle; draw up 5 cc. blood. Loosen the tourniquet and discharge the blood into a 15-cc. centrifuge tube. The venous puncture ordinarily requires no dressing. The procedure is best carried out with the patient lying down. With children, or in cases where a suitable vein is not accessible, the blood may be secured by pricking the finger or ear deeply and collecting the blood in a Wright's or Lyon's tube. The Keidel tube is satisfactory but expensive. When the serum has clotted, separate the clot from the side of the tube, and set in a cool place, to allow the clot to contract. When the serum has separated, pipette off and transfer to another sterile glass container till tested. Keep in the ice box at a temperature not above 6°C. Sera should always be separated from the clot before shipment, as if this is not done more or less hæmolysis will take place en route, rendering the serum unfit for testing.

Serum should be shipped in a sealed glass capsule, or small, sterile, rubber-stoppered bottle. The stopper should be firmly inserted and a strip of adhesive plaster pasted over the top to guard against its being dislodged. Blood serum in sealed glass capsules, or sterile vials, should be well wrapped in cotton and placed in a double mailing case, as specified by the postal regulations.

Just before subjecting the sera to the Wassermann test they should be heated in a water bath at 54° to 56°C. for one-half hour, but spinal fluids do not require heating. The sera should be fresh—i.e., not more than 24 hours old. Tests may be performed with sera older than this, but in that case more negative results with the sera of syphilitics are to be anticipated than if the sera were fresh.

Cerebrospinal fluid.—This is obtained by lumbar puncture according to the method described in the chapter on spinal fluid examination. If the spinal fluid contains blood, it should be centrifuged until clear. It is not to be inactivated before using, as it does not contain haemolytic complement. Possessing weaker deviating powers than blood serum, larger doses are necessary (0.1 cc.; 0.2 cc.; 0.5 cc. and 1.0 cc.).

Standardization of Reagents, Titrating

The Wassermann reaction, properly performed, is a quantitative biochemical reaction, our knowledge of which is wholly empirical. To perform it properly, the various quantities of the elements entering into it should be measured as precisely as possible. Furthermore, it is evident that the substances used are extremely complex organic materials, and the greatest possible care should be taken to handle them properly and measure them accurately. A word may be said here about the use of the graduated pipettes, in measuring reagents diluted or undiluted.

To measure 0.1 cc. use a 1 cc. pipette graduated in tenths.

To measure 0.2 cc. use a 1 cc. pipette graduated in tenths.

To measure from 0.3 cc. to 1 cc. use a 1 cc. or 2 cc. pipette graduated in tenths.

To measure 1 cc., or multiples thereof, use a 5 cc. or 10 cc. pipette graduated in tenths.

In measuring sodium chlorid solution to make up the contents of tubes to unit volume, a 5 cc. pipette graduated in tenths may be used to measure quantities of 0.5 cc. and more.

The necessity for conscientious accuracy in the use of pipettes can not be over emphasized. In measuring reagents the direct measurements of minute amounts (less than 0.1 cc.) is to be avoided. Such amounts should be measured indirectly by diluting the reagent with 0.9 per cent. sodium chlorid solution and measuring a portion of the resulting solution corresponding to the desired amount of the undiluted reagent. In making dilutions the conical test glasses are convenient, and thorough mixing may be secured by blowing air through the solutions. It is needless to remark, after measuring one reagent the pipette should be discarded and a fresh one used before measuring another reagent.

The substances first requiring attention are sheep's corpuscles, hæmolytic amboceptor (rabbit) serum, and complement (guinea pig) serum. The proper adjustment of these substances in relation to each other, known as the adjustment of the hæmolytic system, is an essential preliminary to the tests for syphilis.

Sheep's blood corpuscles.—The sheep cell suspension previously described is added to 0.9 per cent. sodium chlorid solution in the proportion of 5 cc. of the suspension to 95 cc. of the saline solution. (For details of preparation see amboceptor titration and complement titration.) This suspension is taken as an arbitrary starting point in measuring the amount of haemolytic amboceptor present in

the rabbit serum, and the quantity of complement present in the guinea pig serum to determine the proper amounts of these substances to use in the tests.

It should always be remembered, however, that the red blood corpuscles of different sheep vary considerably in the ease with which they are hæmolyzed by complement and amboceptor; so that the substitution of the corpuscles of one sheep for those of another may cause an actual variation of as much as 100 per cent. in the quantity of amboceptor or complement serum necessary to cause complete hæmolysis, and thus give the appearance of a sudden change in the potency of these reagents. This variability of the sheep corpuscles is taken into account and provided for, as is the variability of other reagents, by daily titration of complement just before setting up the tests for syphilis.

Titration of hæmolytic amboceptor.—The "unit of amboceptor" is the smallest amount of amboceptor serum which with 0.05 cc. fresh pooled guinea pig serum will completely hæmolyze 1.0 cc. of the 5 per cent. suspension of sheep cells, when exposed to a temperature of 37°C. for one hour.

Select a specimen of antisheep rabbit serum at least two weeks old. Place 0.1 cc. in a conical glass and add precisely 19.9 cc., 0.9 per cent. sodium chlorid solution (i.e., 1 in 200 dilution); mix thoroughly by blowing air through the solution; then each cubic centimeter of the final dilution will contain 0.005 cc. rabbit serum. Now place the following amounts of the final dilution in a row of test tubes:

0.1 cc. containing 0.0005 cc. amboceptor serum.
0.2 cc. containing 0.001 cc. amboceptor serum.
0.3 cc. containing 0.0015 cc. amboceptor serum.
0.4 cc. containing 0.002 cc. amboceptor serum.
0.5 cc. containing 0.0025 cc. amboceptor serum.
0.6 cc. containing 0.003 cc. amboceptor serum.

Add one tube, containing no amboceptor serum, to the row and make up the volume in all tubes to 2 cc. with 0.9 per cent. sodium chlorid solution. Take 1 cc. of the pooled sera of at least 5 guinea pigs, which has been obtained within 5 hours and kept cold, the serum to be unsalted, and add 19 cc. 0.9 per cent. sodium chlorid solution. Each cc. will therefore contain 0.05 cc. of the guinea pig serum. Add 1 cc. of the diluted guinea pig serum containing complement to all the test tubes. Next add to all the tubes a 5 per cent. dilution in saline solution of the sheep cell suspension already described, making the total volume in each tube 4 cc., mix thoroughly by agitating the tubes, place in the 37°C. water bath for one hour, and keep at about 15°C. overnight. Note the tube containing the least amount of hæmolytic amboceptor serum which shows complete hæmolysis. By complete hæmolysis is meant a cloudless red solution with no undissolved corpuscles at the bottom of the tube. The amount of rabbit serum in this tube is the "unit of amboceptor." Reject, as unsuitable, those specimens of rabbit serum which fail to give complete hæmolysis in amounts of 0.002 cc. or less, with 0.05 cc. pooled complement sera. Amboceptor serum should be retitrated every six weeks. In titrating a new specimen of amboceptor serum set up a duplicate test, using a specimen of amboceptor serum of known titer.

Titration of complement.—This is to be done daily just before the syphilis tests are set up.

The "unit of complement" is the smallest amount of complement serum which, with two units of amboceptor, will completely hæmolyze 1 cc. of the 5 per cent. sheep cell suspension when kept at a temperature of 37°C. for one-half hour.

Estimate, in round numbers, the number of cubic centimeters of red cell suspension needed for the day's work; for example, 100 cc. Multiply the unit of amboceptor by 200 and place that amount of amboceptor serum in a 100 cc. glass-stoppered graduated cylinder. Add about 50 cc. of 0.9 per cent. sodium chlorid solution, taking care to wash down the serum adhering to the sides of the cylinder; next add 5 cc. of the undiluted sheep corpuscles which have been made up to the volume of the defibrinated blood. Then make up to 100 cc. with 0.9 per cent. sodium chlorid solution. Invert 50 times to mix thoroughly. Set aside for 15 minutes.

Dilute some of the salted complement serum as follows:

Serum	0.3 cc.
Water	0.9 cc.
0.9 per cent. solution sodium chlorid	1.8 cc.
Total	3.0 cc.

Set up seven test tubes, adding the following amounts of the above solution to them: 0.6 cc., 0.5 cc., 0.4 cc., 0.3 cc., 0.2 cc., 0.1 cc., 0 cc., using a 1 cc. pipette. Make up the volume in each tube with saline solution to 3 cc. Use a 1 cc. pipette to make up tenths and a 5 or 10 cc. pipette to add the necessary 2 cc. Add to each tube 1 cc. of the amboceptor-corpuscle suspension, incubate in a water bath at 37°C. for one-half hour, and read the unit at once by noting the tube containing the least amount of guinea-pig serum in which the cells are completely dissolved.

Titration of antigen.—In determining the suitability and amount of a specimen of the acetone-insoluble lipoids for use as antigen, the following properties of this substance especially concern us.

1. The property of the antigen of combining with complement in the presence of syphilitic sera.

2. The property of the antigen, in much larger amounts, of combining with complement in the presence of normal sera.

3. The property of the antigen of hæmolyzing the red blood cells.

The first two properties are present to a degree in nearly all antigens, while the third occurs only occasionally and is reason for the rejection of the particular specimen in question.

The quantitative estimation of the first is called the antigenic titration; that of the second the anticomplementary titration. These processes may be combined as follows: Set up two parallel rows of 12 tubes and add to them, in pairs, graded amounts of methyl alcohol solution of the antigen to be tested, leaving one pair without antigen for control—viz., 0.2, 0.16, 0.14, 0.1, 0.08, 0.06, 0.04, 0.02, 0.01, 0.006, 0.004, 0.000 cc. In adding the antigen solution, dilute with 0.9 per cent. sodium chlorid solution. Make up to 2 cc. with 0.9 per cent. sodium chlorid solution. To each tube of one row add 0.2 cc. of known positive syphilitic serum, and to each tube of the other 0.2 cc. of known negative serum. Add to each tube two units of complement, just previously determined as already described, contained in 1 cc. of 0.9 per cent. sodium chlorid solution. This may conveniently be done as follows:

Total number tubes = 24 (allowing for fluid lost in measurement, 25 cc. complement solution will be needed).
Unit of complement = 0.03 × 2 = 0.06.
Then 0.06 × 25 = 1.50.

Take salted complement serum	1.50 cc.	
Water	4.5 cc.	(3 × 1.5)
	6.0 cc.	
0.9 per cent. sodium chlorid solution	19.0 cc.	(25 − 6 = 19)
Total	25.0 cc.	

Then each cubic centimeter of the solution contains two units of complement.

Mix contents of tubes thoroughly, place in 37°C. water bath for one hour. Remove and add to each tube 1 cc. of amboceptor sheep corpuscles, used in determining the unit of complement. Incubate one-half hour and set in a cold place about 15°C. over night.

Note the least amount of antigen completely preventing hæmolysis in the tubes containing positive syphilitic serum. Note the largest amount of antigen not interfering with complete hæmolysis in the negative serum tubes. The best antigen gives a wide margin between these readings.

Now select the unit of antigen for use in the syphilis tests between these values. It should be several times the least amount of antigen completely preventing hæmolysis in the tubes containing positive sera; as other positive sera may be encountered, weaker in the syphilitic reacting substance than the specimen used. On the other hand, the unit should not be more than one-half the largest amount of antigen not interfering with complete hæmolysis in the tubes containing negative serum, as other negative sera may be more anti-complementary than the one used in the test, and false positive reactions might result from the use of too much antigen.

Example of selection of antigenic unit:

With negative serum		With positive serum	
Amount antigen	Hæmolysis	Amount antigen	Hæmolysis
0.2 cc.	None	0.02 cc.	None
0.16 cc.	Partial	0.01 cc.	None
0.14 cc.	Partial	0.006 cc.	None
0.1 cc.	Complete	0.004 cc.	Partial
0.	Complete	0.	Complete

Unit selected = 0.02 cc. (methyl alcohol solution).

When the unit has been selected, place 2 units diluted to 3 cc. in a test tube and add 1 cc. of the cell suspension. Incubate 1 hour at 37°C. Should any hæmolysis occur, the specimen of antigen should be rejected as being hæmolytic.

Technique of Performing the Test

After the units of amboceptor and antigen have been determined, the unit of complement titrated, and the patient's sera heated to 54°–56°C. for one-half hour

on the day of the test, the test may be set up as follows (see diagram): Set up a pair of tubes for each new serum to be tested and for the positive and negative control sera which are to be retested. Add one tube for the antigen control, one for the hæmolytic system control, and one for the sheep corpuscle control. The paired tubes are conveniently placed in two rows, front and back. To each of the front row tubes add one unit of antigen contained in 1 cc. of 0.9 per cent. sodium chlorid solution, and to the antigen control tube add 2 units contained in 2 cc. To dilute the methyl alcohol solution of antigen for this purpose multiply the unit by the number of sera to be examined plus five. Place this amount of the methyl alcohol antigen solution in a conical glass and add sufficient of the diluent to make the total volume equal to the number of specimens to be examined plus five.

Eg. Antigen unit = 0.03.

Number of sera including positive and negative control plus 5 = 40.

$0.03 \times 40 = 1.20$.

Use 1.2 cc. antigen solution and add to it 40 cc. minus 1.2 cc., or 38.8 cc. 0.9 per cent. sodium chlorid solution.

Then, each cubic centimeter of the antigen suspension as made up will contain 0.03 cc. of the methyl alcohol solution.

Now to each pair of tubes, corresponding to the sera which are to be tested, add 0.2 cc. of the sample of serum to the front tube and 0.4 cc. to the back tube. Make the volume of all tubes equal 2 cc. with 0.9 per cent. sodium chlorid solution. To do this add 2 cc. of it to the hæmolytic control tube, 3 cc. to the corpuscle control tube, 1.6 cc. to the tubes in the back row containing sera and 0.8 cc. to the tubes in the front row.

Add to all tubes, save the corpuscle control tube, 2 units of complement contained in 1 cc. 0 9 per cent. sodium chlorid solution (see antigen titration). Add 1 cc. of this mixture to each tube, except the corpuscle control tube. The volume contained in each tube will now be 3 cc. Mix well, by individually agitating each tube. Incubate in a water bath at 37°C. for one hour. Add to each tube 1 cc. of the amboceptor-sheep corpuscle suspension. Mix well and incubate as above for one hour. place in a cool place at about 15°C. overnight.

Reading and Recording Results

The morning after performing the tests, first examine the control tubes. All the red cells in the antigen and hæmolytic system control tubes should be hæmolyzed, but there should be no trace of hæmolysis in the corpuscle control tube. Next examine the tubes containing known positive and negative sera. The rear tubes of both these pairs should show complete hæmolysis, as should the front tube of the pair containing negative serum. The front tube of the positive pair should, however, show little or no hæmolysis, indicating complete, or nearly complete, fixation of complement. In like manner examine all the tubes containing serum to be tested. Inspect the back tubes first; if complete hæmolysis is not present it may be concluded that the serum was anticomplementary, i.e., was capable of fixing complement in the absence of antigen, and that any fixation in the front tube is of doubtful significance. In some sera, in which hæmolysis is complete in the back tube, various degrees of fixation, i.e., weakening of hæmolysis, will be noted in the corresponding

front tubes. If the appearances of the controls previously mentioned are satisfactory, it is permissible to conclude that these sera are positive.

Record the results of the tests, as indicated by the amounts of fixation in these tubes as compared with a specimen showing complete fixation (no hæmolysis) and one showing no fixation (complete hæmolysis) reporting the results as follows:

70 to 100 per cent. fixation = "strongly positive."
40 to 70 per cent. fixation = "positive."
20 to 40 per cent. fixation = "weakly positive."
0 to 20 per cent. fixation = "negative."

In actual practice, with the technique described, the experience has been that nearly all sera give either "strongly positive" or "positive" reactions, or are frankly negative.

Diagram of Complement Fixation Test for Syphilis

The squares represent the arrangement of tubes as seen by one looking down on the rack. Inside the squares appear the reagents in the order in which they are introduced together with the amounts. The preliminary phase of the incubation is carried out at 37°C. for 1 hour. Add amboceptor-cell suspension, incubate at 37° for one-half hour, and keep at about 15°C. overnight.

Back Row

Known positive serum	Known negative serum	Unknown serum[1] to be tested for syphilis	Antigen control	Hæmolytic system control	Sheep corpuscle control
Serum .4 cc. NaCl sol. 1.6 cc. Complement dilution 1 cc. Amboceptor-corpuscle suspension 1 cc.	Serum .4 cc. NaCl solution 1.6 cc. Complement dilution 1 cc. Amboceptor-corpuscle suspension 1 cc.	Serum .4 cc. NaCl solution 1.6 cc. Complement dilution 1 cc. Amboceptor-corpuscle suspension 1 cc.	Antigen suspension 2 cc. Complement dilution 1 cc. Amboceptor-corpuscle suspension 1 cc.	NaCl solution 2 cc. Complement dilution 1 cc. Amboceptor corpuscle suspension 1 cc.	NaCl solution 3 cc. Amboceptor-corpuscle suspension 1 cc.

Front Row

Antigen suspension 1 cc. Serum .2 cc. NaCl solution .8 cc. Complement dilution 1 cc. Amboceptor-corpuscle suspension 1 cc.	Antigen suspension 1 cc. Serum .2 cc. NaCl solution .8 cc. Complement dilution 1 cc. Amboceptor-corpuscle suspension 1 cc.	Antigen suspension 1 cc. Serum .2 cc. NaCl solution .8 cc. Complement dilution 1 cc. Amboceptor-puscle suspension 1 cc.			

[1] But one tube shown in diagram.

The Antihuman Complement Fixation Test (*Noguchi*) for Syphilis

Noguchi replaced the antisheep by an antihuman amboceptor in order to avoid masking a positive Wassermann reaction by the excess of natural antisheep amboceptor present in many human sera. The occurrence of isohaemolytic amboceptor in human sera is comparatively rare and seldom sufficient in amount to cause any disturbance.

Aside from the reagents used in this test the following special apparatus will be needed: Pipettes of 1 cc. capacity graduated to deliver 0.01 cc.; 10 cc. pipettes graduated to deliver 0.1 cc.; small test tubes, the best dimension being 10 by 1 cm.; larger test tubes for complement, antigen, and cell suspension; capillary pipettes (Wright's); test-tube racks with two parallel rows of holes and a centrifuge. A hot-air incubator or, better, a water bath will be needed for incubation.

Physiological salt solution (0.9 of 1 per cent.) should be freshly prepared, boiled, and cooled before use.

The following reagents are used in this test: Blood cell (erythrocyte) suspension; complement; antihuman amboceptor; antigen; blood serum to be tested.

Preparation and Preservation of Reagents

Human blood cell suspension.—The suspension can be prepared from the blood of the patient being examined or from normal individuals. Blood is collected in tubes or flasks containing sodium citrate (2 per cent.) in normal (0.9 of 1 per cent.) salt solution, using approximately 1 cc. of blood to each 9 cc. of the sodium citrate solution. After being thoroughly shaken the mixture is placed in graduated centrifuge tubes and centrifuged four times, the supernatant salt solution being poured or pipetted off each time and being replaced with salt solution. After the last washing the supernatant fluid is removed and enough salt solution added to the *packed cells* to make a 5 per cent. suspension. The cells should be packed thoroughly and the amount noted before the salt solution is added. Each 0.1 cc. of packed cells will make 2 cc. of a 5 per cent. suspension.

Complement.—Guinea pig's serum not more than 36 hours old is used and is obtained in the same manner as described under the Wassermann test. To each 1 cc. of clear serum add one and one-half times the volume of salt solution, making a 40 per cent. dilution.

Amboceptor.—It is the blood serum of rabbits which have been immunized to human erythrocytes. Various methods may be used for immunization, but a method that has given excellent results with extremely low mortality among rabbits is as follows: Large rabbits receive intravenous injections of 5 cc. of a 20 per cent. suspension washed human blood corpuscles at seven-day intervals for 3 injections. Allow a seven-day rest period and then give daily injections of 2½ cc. of 20 per cent. washed blood cells for a period of five days. After a week the rabbit serum will usually contain amboceptor of high titer. If the serum titer should be low, repeat the series of 2½ cc. injections, which will usually bring the serum up to the requisite titer. When the preliminary titration to be described shows that the serum contains sufficient haemolysin the animals are bled and the serum preserved as described under the Wassermann test.

Antigen.—The acetone-insoluble fraction of tissue lipoids described under the Wassermann test is used. Experience has shown that a suitable acetone-insoluble antigen accomplishes all that is possible, and is applicable with both the inactivated and the unheated serum.

Emulsion of antigen.—The method of adding the salt solution to the stock antigen is very *important.* The amount of stock antigen to be used in a series of tests (0 1 cc. of antigen and 0.9 cc. of saline is sufficient for 10 tests) is placed in a test tube and the salt solution added drop by drop, the mixture being shaken thoroughly each time a drop is added. The antigenic value of the same sample of antigen may differ greatly according to the manner of preparation of the saline emulsion. If the antigen be added to the salt solution or the salt solution be rapidly added to the antigen a faintly bluish white emulsion is obtained, while mixing drop by drop we get a whitish opaque emulsion. These two emulsions by actual titration will vary greatly in their antigenic power; the milky opaque emulsion (made by thoroughly shaking) will be about four times as strong as the opalescent emulsion (rapid mixing) for the same specimen of syphilitic serum.

Collection of human serum.—Blood is collected for the test as described under the Wassermann test.

Titration of the Materials Used in the Test

The amboceptor and antigen.—Owing to temperature changes and other factors, should be titrated carefully at frequent intervals. While the complement is variable the amboceptor is more or less constant and the former should be titrated against a definite amount of amboceptor. This should be done before each series of tests. While complement is variable it will rarely be met with, and complement-deficient guinea pigs should be eliminated from breeding stock.

Titration of complement.—The titration should be made as illustrated in the following table, using the standard (5 per cent., cell suspension, amboceptor of known strength, and 40 per cent. dilution of the guinea pig serum.

Titration of Complement

Tube No.	Amount of complement	Amount of saline	Amount of amboceptor (units)	Amount of 5 per cent. cells
1	0.03	0.9	2	0.1
2	.04	.9	2	.1
3	.05	.9	2	.1
4	.06	.9	2	.1
5	.07	.9	2	.1
6	.08	.9	2	.1
7	.1	.9	2	.1
8	.1	.9	0	.1

Incubate in water bath at 37°C. for one-half hour or the bacteriological incubator for one hour. The tubes are shaken every ten minutes to liberate the amboceptor from the paper in which it is impregnated. After incubation the first tube showing complete haemolysis is noted and the amount of complement in it is called the complement unit. Tube No. 3 containing 0.05 cc. of complement usually will show complete haemolysis and would constitute one unit of complement and in making the test twice this amount or 0.1 cc. would contain two units. Tube No. 8 containing no ambocepter should show complete inhibition or absence of haemolysis.

Titration of ambocepter.—Preliminary titration of the serum of immunized rabbits is made in the following manner: The blood is collected from the rabbit's ear in a Lyon or Wright capsule, the serum separated and inactivated at 56°C. for 30 minutes. Prepare a 1 to 100 dilution (0.1 cc. of serum and 9.9 cc. salt solution) and increasing amounts of this dilution are then placed in tubes as follows: 0.05, 0.1, 0.15, 0.2, 0.25 cc. To each tube is added 0.1 cc. of 5 per cent. red cell suspension and two units of complement and sufficient salt solution to bring up the volume of each tube to 1 cc. An amboceptor suitable for placing on paper will give complete haemolysis in the tube containing 0.1 cc. of the 1 to 100 dilution or a titer strength of 1 to 1000. If it is not, the immunization is continued. If haemolysis is complete the rabbit is bled, and the blood serum, after inactivation at 56°C. for 30 minutes, is placed on Schleich and Schulls No. 597 filter paper, the paper being cut in pieces 10 by 10 cm. and thoroughly impregnated. The impregnated paper is dried and cut in strips 5 mm. wide and various lengths are used in titrating against the previously standardized complement. We have found that this paper keeps better, especially in the tropics, if preserved in a tightly stoppered container with a small amount of $CaCl_2$ in the bottom.

Titration of Amboceptor Paper

Tube No.	Amount of complement (units)	Amount of saline	Millimeters of amboceptor paper	Amount of 5 per cent. cells
1	2	0.9	5 by 1	0.1
2	2	.9	5 by 2	.1
3	2	.9	5 by 3	.1
4	2	.9	5 by 4	.1
5	2	.9	5 by 5	.1
6	2	.9	None	.1

Incubate in water bath or incubator as for complement titration, shaking every 10 minutes to liberate the amboceptor serum, and after 30 minutes the titration is read. The first tube showing complete haemolysis is noted and the amount of paper contained in that tube is called one unit of amboceptor. A good paper should show complete haemolysis in tube 2 or 3. In making the test two units of paper are used.

For instance, if one unit was found by titration to be 5 by 2 mm., a piece 5 by 4 mm. should be used in the test.

Titration of the antigen.—The antigen should be titrated to determine its haemolytic, anticomplementary and antigenic properties. Before titration the antigen is diluted as described.

TITRATION OF ANTIGEN FOR ANTICOMPLEMENTARY PROPERTIES

Tube No.	Amount of complement (units)	Amount of saline	Amount of antigen 1:10
1	2	0.9	0.05
2	2	.9	.1
3	2	.9	.15
4	2	.9	.2

Incubate for 30 minutes at 37°C. in water bath or incubator for one hour and add to each tube 2 units of amboceptor and 0.1 cc. of the 5 per cent. red cell suspension.

Repeat incubation, shaking tubes every ten minutes.

After the second incubation the titration is read and all of the tubes should show complete haemolysis. If any one of the four tubes shows inhibition of haemolysis the antigen is anticomplementary and should not be used in the test.

TITRATION OF ANTIGEN TO DETERMINE ANTIGENIC PROPERTIES

Tube No.	Amount comp. units	Amount of luetic serum	Amount of saline	Amount of antigen 1:10		Amount of 5 per cent. cells	Units amboceptor
1	2	0.1	0.9	0.05	Incubate at 37°C. in water bath, or 1 hour in incubator	0.1	2
2	2	.1	.9	.10		.1	2
3	2	.1	.9	.15		.1	2
4	2	.1	.9	.20		.1	2
5	2	.1	.9	None		.1	2
6	2	*.1	.9	.20		.1	2
7	2	*.1	.9	None		.1	2
8	2	None	.9	None		.1	2

* Normal serum.

As a result of this titration, tubes 1, 2, 3 and 4, containing a known syphilitic serum and the antigen should show complete inhibition of haemolysis. Tube 1 sometimes shows a slight trace of haemolysis, and if only slight the antigen may be used. Tube 5, the control of the syphilitic serum, should show complete hæmolysis. Tube

6, containing normal blood serum and antigen, should show complete haemolysis. Tube 8, the control of the haemolytic system, should show complete haemolysis.

The smallest amount of the antigenic emulsion that gives complete inhibition of haemolysis with positive syphilitic serum is called the antigenic unit, and this amount *is not doubled*, as is the unit of complement and amboceptor.

Technique of Performing the Test

For each serum to be tested two tubes are necessary, an anterior and posterior one. In addition there must be a control set of two tubes for known positive serum and a control set of two for known negative or normal serum. Only one set of controls are necessary, no matter how many sera are to be tested. It is also well to have a control tube for the haemolytic system and one for the antigen.

In making the test proceed as follows: In each of the tubes mentioned place 0.9 cc. of salt solution (0.9 per cent.). In tube 1, anterior, place 0.1 cc. of the serum to be tested and the same amount in tube 1, posterior. In tube 2, anterior and posterior, place 0.1 cc. of known syphilitic serum, and in tube 3, both anterior and posterior, place 0.1 cc. of known normal serum. *Add to each tube two units of complement* and one unit of the antigen (usually 0.1 cc. of a 1 to 10 dilution) to each *anterior* tube. Incubate all the tubes in the water bath at 37°C. for 30 minutes. At the expiration of this time add to each tube *two units* of amboceptor paper and 0.1 cc. of 5 per cent. suspension of blood corpuscles. Incubate at 37°C. for 30 minutes, shaking the tubes every 10 minutes to liberate the amboceptor serum from the paper, place in refrigerator for one-half hour and read the results.

The tubes without antigen (back row) should show complete haemolysis, likewise the haemolytic system control set and the negative control set. The front tube of the positive control set should show complete inhibition of haemolysis. With conditions in the control tubes satisfactory, complete inhibition in the front tube of the set containing the unknown serum indicates a positive reaction (++++), while complete haemolysis indicates a negative reaction. If haemolysis is almost completely inhibited, the test is still positive (+++), but in the case of 50% (++) or 75% haemolysis (+) the result should be regarded as doubtful unless syphilis has been clinically established, or as an indication for further treatment if the patient is known to be syphilitic.

In the event there is complete inhibition in both the anterior and posterior tubes containing the patient's serum, the reaction is regarded as anticomplementary and the test should be repeated with another specimen of serum.

A very weak or doubtful reaction may sometimes be obtained in the case of undisputed syphilis. In such an instance the test should be repeated with 0.15 and 0.2 cc. of inactivated serum. For examining the serum from known cases of syphilis for prognostic purposes the test must be made with 0.15 and 0.2 cc., in case the routine amount no longer gives a positive reaction. In all cases it is a wise precaution to take blood for examination shortly before meal-time, and alcohol should be denied the patient for at least 24 hours previous to taking the blood.

The following table illustrates the method of making the test:

Serum for diagnosis	Positive control	Negative control
Anterior tubes	Anterior tubes	Anterior tubes
Patient's serum 0.1 cc. Complement 2 units Antigen 0.1 cc. Saline 0.9 cc.	Positive serum 0.1 cc. Complement 2 units Antigen 0.1 cc. Saline 0.9 cc.	Normal serum 0.1 cc. Complement 2 units Antigen 0.1 cc. Saline 0.9 cc.
Posterior tubes	Posterior tubes	Posterior tubes
Patient's serum 0.1 cc. Complement 2 units Saline 0.9 cc.	Positive serum 0.1 cc. Complement 2 units Saline 0.9 cc.	Normal serum 0.1 cc. Complement 2 units Saline 0.9 cc.

Incubate for one-half hour 37°C., in water bath or for one hour in incubator. Add two units of amboceptor paper and 0.1 cc. of 5 per cent. red cell suspension to each tube.

Titration of the Antibody Content of the Syphilitic Serum

In order to measure accurately the exact strength of the antibody content of the patient's serum it may be titrated, as a syphilitic serum may give complete fixation with less than 0.1 c.c.—that is, we are able to distinguish the intensity of the reaction or determine the number of fixing units present in a given specimen of serum.

To titrate the strength of inactivated serum prepare a 1 to 10 dilution by diluting 0.5 cc. of serum with 4.5 cc. of saline. Each tube will contain the patient's serum in varying amounts as shown in the following table:

AMOUNT OF DILUTED SERUM

Tube No. 1.....1.0 cc. (1:10) equal to 0.1 cc. inactivated serum.
Tube No. 2..... .5 cc. (1:10) equal to .05 cc. inactivated serum.
Tube No. 3..... .4 cc. (1:10) equal to .04 cc. inactivated serum.
Tube No. 4..... .3 cc. (1:10) equal to .03 cc. inactivated serum.
Tube No. 5..... .25 cc. (1:10) equal to .025 cc. inactivated serum.
Tube No. 6..... .2 cc. (1:10) equal to .02 cc. inactivated serum.
Tube No. 7..... .15 cc. (1:10) equal to .015 cc. inactivated serum.
Tube No. 8..... .1 cc. (1:10) equal to .01 cc. inactivated serum.

Having measured the amounts of serum into the tubes and brought the volume of the fluid in each tube up to 1 cc. by uniformly adding salt solution, one now makes the test in the usual way. Add two units of complement and 0.1 cc. of the standard

antigen emulsion. Mix the contents well and incubate in water bath at 37°C. for 30 minutes. At the end of the incubation add to each tube 0.1 cc. of 5 per cent. red blood cell suspension and two units of amboceptor paper, and repeat incubation. If the specimen gives complete fixation in the first tube (1 cc.), it is said to contain one unit of antibody. If it occurs in the second tube (0.5) it contains two units, etc. A specimen showing complete fixation in the last tube (0.1 cc.) must contain at least 10 antibody units and may be further titrated to find out its real titer.

Cerebrospinal fluid.—Examination of the cerebrospinal fluid differs from that used for inactivated sera only in one respect, that of the quantity of the specimen used, which may vary fron 0.1 to 0.5 cc. No inactivation is required, as the cerebrospinal fluid contains no complement.

Homohaemolytic System for the Serum Diagnosis of Syphilis. (*Noguchi*)

Under ordinary circumstances the antihuman haemolytic system, with guinea-pig complement, is most satisfactory, but in emergencies in which guinea-pig serum is not available (on board ship and isolated stations) a reliable diagnosis can be made by substituting human for guinea-pig complement. In certain exceptional cases where the complement content of the serum is below the average, special adjustment of the haemolytic system must be made. Even this disadvantage, however, is removed by the use of a definite amount of normal human complement with inactivated serum.

Preparation of reagents.—The details with regard to the reagents used in conducting the homohaemolytic system are essentially the same as those described in connection with the heterohaemolytic system using guinea-pig complement.

The red blood cell suspension is prepared as previously described. Amboceptor paper and antigen are prepared and titrated as outlined.

The patient's serum is collected and kept in the ice box. Serum more than 24 hours old should not be used, unless inactivated by heating to 56°C. and the complement furnished by the addition of known normal human serum.

Titration of amboceptor.—In carrying out the homohaemolytic system it is necessary to find the minimum hæmolytic dose of antihuman amboceptor paper which will cause complete haemolysis in the presence of 0.2 cc. of fresh human serum of 0.1 cc. of 5 per cent. human blood cell suspension in 15 minutes in the water bath at 37°C. Two amboceptor units are used in the test. The only difference between the action of the human and guinea-pig complement lies in the fact that the former requires more antihuman haemolytic amboceptor to render it active against the human corpuscles than the latter.

Positive and negative controls as in any other serodiagnostic procedure must accompany the serum to be tested. Where many tests are being made daily or every other day the necessary positive and negative sera will be furnished by the tests of the previous occasion.

PROCEDURE FOR EXAMINING FRESH HUMAN SERA BY HOMOHAEMOLYTIC SYSTEM

Serum for diagnosis	Positive control	Negative control
Anterior tubes	Anterior tubes	Anterior tubes
Patient's serum 0.2 fresh Antigen 0.1 cc. Saline 0.9 cc.	Positive serum 0.2 fresh Antigen 0.1 cc. Saline 0.9 cc.	Normal serum 0.2 fresh Antigen 0.1 cc. Saline 0.9 cc.
Posterior tubes	Posterior tubes	Posterior tubes
Patient's serum 0.2 fresh Saline 1.1 cc.	Positive serum 0.2 fresh Saline 1.1 cc.	Normal serum 0.2 fresh Saline 1.1 cc.

Incubate for 30 minutes at 37°C. in water bath or 1 hour in incubator.

Add 2 units amboceptor to each tube and 0.1 cc. of 5 per cent. red cell suspension. Repeat incubation for same period.

Shake tubes every 10 minutes after the addition of amboceptor paper to liberate the amboceptor serum. Read results after the tubes have stood at room temperature for 30 minutes. Readings of the test are made in the same manner as in the test using guinea-pig complement.

Procedure for the Examination of Sera More than 36 Hours Old

It is best to inactivate all sera more than 36 hours old by heating to 56°C. for 20 minutes, as specimens which have stood for this length of time in the refrigerator are inconstant in their complementary activity. Negative fresh human serum containing active complement is added to the inactivated serum. *For cerebrospinal fluid* 0.2 cc. of fresh active negative human serum is added as complement.

Refer to the table illustrating the procedure for making the test with guinea-pig serum. Instead of the two units of complement used, 0.2 cc. of active human serum is added and 0.2 cc. of the serum of the patient. A complete test of the active negative serum used as complement should accompany the test as a negative control.

Specimens which fail to completely haemolyze in the control tube may be deficient in complement, and to these sets of tubes, both the anterior and posterior, another amboceptor unit may be added, the tubes placed in a separate rack and subjected to further incubation. Should the specimen still fail to haemolyze completely in the control tube, even with additional amboceptor, the serum should be tested again, by adding to it a quantity of fresh serum which has been shown to contain an average complement and at the same time to be devoid of syphilitic fixing substance (negative serum).

Results with this method correspond closely with the original Noguchi method in which guinea-pig complement is used. Occasionally weak positive or negative reactions are obtained with sera containing an excessive amount of complement.

If complete haemolysis takes place in five minutes it is obvious that there is an excess of complement in the specimen, and if a negative or weakly positive reaction is obtained with such a serum the test should be repeated, the amount of serum used being reduced to such an extent that complete haemolysis takes place in the control tube in 15 minutes. Finally, it should be emphasized that only the acetone-insoluble (Noguchi) antigen should be used, as nonspecific positive reactions are encountered when cholesterin-fortified and plain alcoholic extracts are used as antigens.

Complement Fixation Technic as Applied to the Kahn Test

This procedure gives equally good results with the antihuman and antisheep haemolytic systems. In the laboratories of the U. S. Naval Medical School, Houghton, has employed this technic with excellent results. It demonstrates the essential unity of the two reactions, showing that the phenomena of precipitation and complement fixation are closely related (Kahn).

The reagents required are the same as those described in the Wassermann and Noguchi procedures. If the antisheep system (Wassermann) is employed the titrations are carried out in the same manner using $\frac{1}{10}$th quantities of complement (10%) and sheep cell suspension (0.1 cc.). For the antihuman system the reagents are used in the same quantities as given in the Noguchi test.

Following the completion and recording of results of the routine Kahn test, the front and center rows of tubes are discarded; i.e., the tubes containing the 3:1 and 6:1 serum-antigen concentrations, leaving the back tube containing 12:1 concentration. Replace the front and center tubes with clean Kahn tubes. Disregarding the quantity of antigen (0.0125 cc.) in the back tube, we have 0.15 cc. of serum and 0.5 cc. of salt solution, making a total volume of 0.65 cc. Now to the back row of tubes add 0.05 cc. of salt solution increasing the total volume to 0.7 cc. From the back row of tubes remove 0.3 cc. (0.06 cc. serum-antigen mixture) and place in center tube, leaving 0.4 cc. (0.09 cc. serum-antigen mixture) in the back tube. In the front tube place 0.1 cc. of the patients serum. Make up the total volume of all tubes to 0.4 cc. with normal salt solution, and add two units of complement (usually 0.1 cc. of 10%) to all tubes. Shake well to mix ingredients and incubate in the water bath at 37°C. for 30 minutes. Then add two units of amboceptor and the washed red cell emulsion. Shake well and place in the water bath for another 30 minute incubation period and the results are read after placing in the ice box for a half hour or the following morning. The primary fixation may be carried out in the ice box (8°C.) with the antisheep system, but this has not been satisfactory with the Noguchi method, where the water bath should be employed. The same procedure is applicable to spinal fluids.

General Considerations

According to Stokes the highest positive efficiency of the Wassermann test is reached in a period extending from about three weeks

after the appearance of the chancre to the height of secondary manifestations, covering roughly about six months. From this pinnacle of efficiency the proportion of positive Wassermann results obtained from the blood gradually declines.

In the primary stage Stokes states that the proportion of positive blood Wassermann reactions at the beginning of the second week averages about 35 to 50%, from which point it increases steadily to 80% or more at the end of the eighth week.

McDonagh states that in the primary stage of syphilis the blood Wasserman is positive in 40% of cases. Noguchi gives 69.8% as the average determined by various serologists.

Craig gives the following summary of positive blood Wassermanns: First week 36.3%, second week 59.3%, third week 68.9%, fourth week 77.2%, fifth week 81.3%.

In frank secondary syphilis Stokes found that taking all cases as they came, treated and untreated, 92% were positive, while in the untreated cases 98.5% gave the positive reaction.

In untreated secondary cases McDonagh states that 97% give positive results, while Noguchi's average, as determined by various serologists is 89.4% positive.

Stokes and McFarland in early secondary syphilis found the following: blood positive and spinal fluid negative 40%, blood positive and spinal fluid positive 36%, blood negative and spinal fluid positive 8% and blood and spinal fluid negative 16%.

Fordyce and Rosen give the following percentages in late secondary syphilis; blood positive and spinal fluid negative 26%; blood positive and spinal fluid positive 21%; blood negative and spinal fluid positive 5% and blood and spinal fluid negative 47%.

Late syphilis.—In the experience of Stokes, the blood is negative in as high as 59% of the neurosyphilitics in general. In tabes Nonne found the blood negative 30 to 40% and Gennerich in 50 to 60%. In paresis it is generally accepted as positive in 90 to 100%, although Kafka has reported as high as 18.7% negative blood Wassermanns in this disease.

In the tertiary stage McDonagh states that the blood Wassermann is positive in about 70%. Noguchi's averages, as determined by various serologists for positive blood Wassermann reactions are: Tertiary syphilis 78.1%, cerebrospinal syphilis 47.6%, paresis 88.1%, tabes 62.6% and for cerebrospinal fluid: cerebrospinal syphilis 19%, paresis 90% and tabes 56.2%.

Nonne obtains from 85 to 90% positive Wassermann reactions on 0.2 cc. of spinal fluid in paresis as against 20% in tabes and 29 to 30% in cerebrospinal syphilis. On the other hand, with 1 cc. of spinal fluid almost all tabetics yield positive Wasserman reactions; paretics and taboparetics are invariably positive, and patients with cerebrospinal syphilis yield almost 100% positives.

In regard to both blood and spinal fluid findings in late syphilis Stokes and Brown give the following figures: blood positive spinal fluid negative 23%; blood and spinal fluid positive 18%; blood negative and spinal fluid positive 41%; blood and spinal fluid negative 18%. In the same stage of the disease Fordyce and Rosen report: blood positive spinal fluid negative 22%; blood and spinal fluid positive 48%; blood negative and spinal fluid positive 14% and blood and spinal fluid negative 16%.

For further comparison of the blood and spinal fluid Wassermann in late syphilis see chapter on spinal fluid examination.

In a survey of untreated preponderantly late syphilis Des Brisay obtained the following positive blood Wassermann reactions: visceral 100%, latent 93.8%, cardiovascular 85%, osseous 84.2%, cutaneous 81.2%, mucous membrane 80%, neurosyphilis 41%. Stokes found that in all types of syphilitic lesion of the vascular system, treated and untreated, 57.5% gave a strongly positive reaction on first test. Repetition, provocative procedure or other measures yielded an additional 10%.

Heredosyphilis.—Cooke and Jeans found that at birth syphilitic infants give a negative blood Wassermann in 37%, weak positives in 18%, and a strong positive in 45%. Ross and Wright observed the delay of a month or more before many syphilitic infants became Wassermann positive. After several months all syphilitic infants show strongly positive reactions. Cruikshank states that non-syphilitic infants may give a weakly positive reaction at birth which becomes negative later. For this reason Fordyce and Rosen advise taking the infant's blood on the tenth day after birth. Stokes cliams that in heredosyphilis the diagnostic value of the blood Wassermann reaction in the second and third months is 100%; in the first decade 88%; second decade 63%; third decade 46%; and after thirty years 15% at which time half of the cases are identified by clinical signs.

Noguchi's average, as determined by various serologists, for hereditary syphilis is 94.5%.

Day and McNitt, working under Dock, have given very valuable data in connection with relation of the Wassermann test to syphilis. The results are based on a study of 2925 cases. By eliminating weakly positive reactions where cholesterolized antigen was used (in the absence of positive clinical evidence) they found 6.3% of the higher social class, 13.6% of those in pay wards, 19.8% of patients in free wards and 30% of colored patients as positive for lues. Of cases with a negative clinical history, 16.9% gave strongly positive reactions; with a suspicious history 22.5%, and with a positive history 61%.

In 268 cases at the medical clinic of Johns Hopkins Hospital, Clough failed to obtain a positive reaction in 99 cases which were negative clinically.

In 45 cases of syphilis he obtained 73% of positive results. Excluding cases which had received thorough treatment 82% were positive. Tabes gave 40% and general paresis 100%. In five cases of primary syphilis four gave positive reactions. Kolmer gives 96% positives for untreated active tertiary syphilis with 75 to 80% for latent tertiary syphilis; in untreated congenital syphilis of children over one year of age, 97 to 100%. Comparing the luetin reaction with the Wassermann, Noguchi gives 80% for tertiary and 70% positive for congenital syphilis.

Certain cases of syphilis exhibit a persistently positive Wassermann, notwithstanding treatment that is considered standard practice. Stokes and Busman state that this phenomenon seems to accompany grave rather than trivial syphilis.

Of 458 syphilitic patients who had received from twelve to twenty-nine arsphenamine injections combined with mercurial inunctions, the average being fourteen injections and ninety inunctions in eleven months, 6.6% of primary and secondary

cases, and 22% of latent, late and hereditary cases (average duration thirteen years) remained persistently Wassermann-positive. Cardiovascular changes are apparently those most likely to underlie a resistant positive Wassermann test in late syphilis (44%), with neurosyphilis 30%, osseous lesions 30%, hepatic, splenic, and gastric syphilis 21%, and other types from 10 to 17%.

Positive Wassermann reaction in diseases other than syphilis.—In frambesia or yaws, a positive Wassermann reaction seems to be given in a higher percentage than is true for syphilis. Among other diseases reported as having, at one time or other, given a positive Wassermann reaction, Stokes names: lepra, tuberculosis, the acute exanthemata, pneumonia, septicemia, trypanosomiases, relapsing fever, general anesthesia, advanced malignant cachexia, especially hepatic; pernicious anemia, malaria, pregnancy, Weil's disease, diabetes and systemic mycotic infections.

Bacterial Complement Fixation Tests

The two bacterial complement fixation tests which are used as routine diagnostic methods are those for gonorrhoeal and glanders infections.

Similar tests may be made for tuberculosis, typhoid fever, cholera, diphtheria, cerebrospinal fever, pertussis, streptococcal infections and several other diseases of bacterial origin. In many of these diseases, however, agglutination reactions or other immunity reactions, or blood cultures, are less complicated and not so time-consuming and therefore more practical. Veterinarians consider the test of value in contagious abortion of cows and mares.

The best known methods for preparing bacterial antigens are the following:

1. Emulsify the growth on agar, or special medium (for gonococcus), in salt solution, as described under preparation of vaccines. Heat the emulsion at 60°C. for one or two hours and then count the organisms as for vaccines.

For gonococcus test we use an antigen with 4,000,000,000 organisms in 1 cc. This may be used directly as antigen or it may be shaken up with glass beads for several hours to complete disintegration. The antigen can be preserved by the addition of 0.25% trikresol or 0.5% phenol. For glanders one may use a seventy-two-hour culture in glycerin bouillon, sterilized at 60°C. for two hours and preserved with 0.5% phenol.

2. Besredka and Gay prepare their antigen by precipitating the saline bacterial emulsion, washed off agar, with an equal amount of absolute alcohol. Then centrifugalize, pipette off supernatant fluid and dry the sediment *in vacuo* over sulphuric acid. The dried sediment is made into a 2% suspension with isotonic salt solution. For use this stock solution is diluted. There are also methods in which the bacterial sediment is frozen with carbon dioxide snow and then triturated with crystals of sodium chloride so as to make an isotonic saline emulsion. Bacterial sediments can also be dried in calcium chloride desiccators.

3. Wash off agar growth with 50% alcohol and centrifuge. Pipette off supernatant alcohol. Add 50% alcohol and let stand for 30 minutes at 37°C. and cen-

trifuge again. Pipette off supernatant alcohol. Then add ether and after shaking let stand 1 hour at room temperature. If the ether is yellow wash again with ether. Then allow to stand until sediment is perfectly dry. The white powder obtained is to be stored in tightly corked tubes. To use suspend one gram of the powder in 200 cc. of physiological salt solution.

In carrying out bacterial complement fixation tests we use an amount of antigen which will by its antigenic power alone fix complement or, as is often stated, be anticomplementary.

Then use one-half this amount as the antigen content for the test.

The method of Noguchi, as previously described, but using one-half the anticomplementary dose of antigen, is satisfactory after experimenting with the proper amount of inactivated serum of the patient to be examined.

For the *Gonococcus* fixation test it is most important to have antigen prepared from a mixture of several strains of gonococci, preferably 10 or 12.

Complement fixation tests have likewise been used in the diagnosis of the following animal parasitic diseases: Echinococcus infection, infection by various trematodes and trypanosomiases (dourine, etc.).

In all of the complement fixation tests previously described, the antigens were known and the antibody unknown. This order may be reversed so that by using a known antibody, an unknown antigen may be determined, as in the identification of blood stains, bacterial antigens, meats, carcinoma, etc.

Determination of Opsonic Power and the Preparation of Vaccines

Determination of the opsonic index, while not used in clinical work so extensively as formerly, is still routinely employed in many laboratories, particularly for tests of immunization and determinations of strain.

In references to the mechanism of immunity contained in the previous pages only the theories of Ehrlich have been brought out. In order to understand the problems involved in the study of opsonins the phagocytic theory of immunity brought forward by Metchnikoff must be studied. Ehrlich's views would seem to hold with diseases where there is an increase in bacteriolytic or antitoxic power of the serum while in such diseases, as are caused by pathogenic cocci, the phagocytic element is operative as there is an absence of bacteriolytic power in the serum of the person with the infection.

There are two kinds of phagocytes, the microphages (represented by the polymorphonuclears) which, on phagolysis or disintegration, give off microcytase, a substance identical with complement or alexine and chiefly bactericidal; and the macrophages (represented by the large mononuclears of the blood and the fixed connective-tissue cells) which exert their action on protozoa or animal cells.

Phagocytes may either act by ingesting bacteria and destroying them intracellularly or they may as a result of phagolysis bring about bacteriolysis extracellularly. According to Metchnikoff the intracellular bacteriolysis explains why an individual may possess immunity yet his serum fail to show any bacteriolytic power.

The control of vaccine treatment by taking opsonic indices from time to time does not seem to have met with much favor in this country—the sources of error being as great if not greater than ordinary variations in the opsonic index during the negative and positive phases.

Method of Wright for Obtaining Opsonic Index

While other observers had previously noted the presence of substances in immune sera which so acted on the bacteria that phagocytosis was made possible, yet it was due to Wright and Douglas, in 1903, that the existence of this factor in phagocytosis was brought forward and its estimation made practicable.

To this substance the name *opsonin* was given—the Greek word from which it is derived indicating preparation of the food—that is, the opsonin so alters or sensitizes the bacteria that they can be engulfed or phagocytized by the polymorphonuclear leukocytes (the microphages of Metchnikoff). About the same time Neufeld and Rimpau noted the presence of a substance in immune sera which so acted on bacteria as to prepare them for phagocytosis. Their designation "bacteriotropic substance" is practically synonymous with opsonin.

In 1902 Leishman introduced the method of determining the "phagocytic index." By taking 1 part of blood and 1 part of an emulsion of the bacteria in question and keeping the mixture in a moist chamber at body temperature for a standard time, as fifteen to thirty minutes, and then spreading the blood-bacteria mixture and staining the film with Leishman or Wright's stain he counted the number of bacteria in a certain number of polymorphonuclears, and by dividing obtained the average number of phagocytized bacteria per leukocyte.

The Wright technique for determining the phagocytic average, and from this the opsonic index, is as follows:

Blood is taken from the patient and at the same time from a normal individual, or preferably the blood of several normal individuals is pooled. This blood is best collected in a Wright's tube, although it may be received in a small test tube. After coagulation and separation of the serum, the serum is ready for use.

The next step is to prepare the leukocyte emulsion. For this we fill a centrifuge tube with normal salt solution, to which has been added 1% sodium citrate —the latter to prevent coagulation. Then having pricked a finger congested by a constricting rubber band, from 15 to 20 drops of blood are added to the citrated salt solution, and the mixture thoroughly shaken. After centrifugalization for about five minutes the red corpuscles will be thrown to the bottom of the tube with the leukocytes forming a superimposed layer. In order to free the leukocytes entirely from serum admixture, the supernatant citrated salt solution is pipetted off, and a fresh tubeful of salt solution is added to the blood-cell sediment. Again shaking, we centrifuge, obtaining for a second time a sediment of blood cells with the leukocytes in the superimposed layer. In some laboratories the washing in salt solution is again repeated, but for all practical purposes two washings as described above suffice.

The superimposed layer of white cells may now be pipetted off from the heavier red cells (of course, containing a large admixture of red cells) to be used as a leukocyte cream—or by slanting the centrifuge tube we can pipette off the proportion of the leukocyte mixture needed from the bottom, sides or top of the slanted layer of blood cells.

Having prepared our leukocyte emulsion, and the serum from the normal individual as well as that from the patient, it only remains to prepare our bacterial emulsion. For bacteria in general, with the exception of tubercle bacilli, we simply take up a small loopful of a young agar culture (eighteen hours or less), and emulsify it uniformly with salt solution, added by degrees until the suspension amounts to 0.5 to 1 cc., and there remains only a faint turbidity. To thoroughly distribute the bacteria, and especially to break up clumps, repeated suction and ejection with a capillary pipette provided with a rubber nipple is satisfactory.

The presence of clumps in a bacterial emulsion invalidates the estimation of phagocytosis, for the reason that a leukocyte will take up a clump of twenty or more bacilli as readily as one organism.

Having at hand (1) the suspension of leukocytes, (2) the bacterial emulsion, and (3) the sera of the patient and the normal individual, we are ready to proceed with the test.

Using a capillary bulb pipette, with a pencil mark to indicate 1 volume, we draw up to the mark the leukocyte cream. Then wiping off the tip of the pipette we raise the column of leukocyte emulsion about ½ inch to make an air break, and then draw up 1 volume of the bacillary emulsion. Again making an air space we draw up 1 volume of the serum of the normal individual. This gives three columns in the capillary tube separated by air spaces. We next eject the three constituents into a watch glass and thoroughly mix them by alternate suction and ejection with the tube and nipple. When mixed we draw the mixture up into the same capillary tube, seal off the capillary end in the flame and put in an incubator for exactly fifteen minutes.

We next repeat the process in the same manner except that the patient's serum is used instead of that of the normal individual.

These tubes having been kept at the same temperature for the same length of time are then taken out, the contents blown into watch glasses, mixed thoroughly a second time, and then smears are made—a drop of mixture being deposited on

a very clean slide and the smear made by Daniel's method as described in "Blood Preparations," but using a spreader-slide narrowed by cutting off one corner. The smears are then stained (Leishman's or Wright's blood stain or Ziehl-Neelsen's for tubercle bacilli) and the number of the bacteria in from 50 to 100 leukocytes counted. This number divided by the number of cells gives the phagocytic average.

The phagocytic average of the patient's tube divided by that of the normal individual's tube gives the opsonic index. Thus, in counting 100 cells we find 500 phagocytized cocci in the patient's tube, giving an average of 5, and in the normal individual's blood we get 1000, an average of 10. Then the opsonic index would be $5 \div 10$, or 0.5.

Preparation of Vaccines

It has been found satisfactory to make use of stock vaccines in the treatment of gonorrhoeal and tuberculous affections. In treatment of tuberculosis Wright prefers Koch's T. R. or New Tuberculin in doses of from $\frac{1}{5000}$ to $\frac{1}{800}$ mg. Some prefer Koch's more recent bazillen-emulsion. It is preferable in the treatment of gonorrhoeal infections, and essential in treating most other infections, that the organism used in the preparation of the vaccine should have been isolated from the patient (*autogenous vaccine*).

In the making of vaccines all media and apparatus should be sterilized with scrupulous care to avoid the danger of tetanus infection. Having isolated the organism, it is inoculated upon one or more agar slants, and after a growth of from five to seven hours, with streptococci and pneumococci, or of eighteen hours for staphylococci and colon, the growth on these inoculated slants is taken up with salt solution, thoroughly shaken up in the diluting solution and standardized. (Esmarch roll of nutrient agar may be inoculated for growing cultures for vaccines.)

The most practical way is to gently rub off the growth on the agar in about 1 or 2 cc. of salt solution with a platinum loop or sterile cotton swab. Then pour the bacterial emulsion into a sterile test tube and repeat the process with three to five agar slants, until we have from 6 to 10 cc. of the emulsion in the sterile test tube. By heating to melting point in the flame a piece of glass tubing and attaching it to the rim of the test tube (also melted), we have a handle with which to draw out the test tube when heated about 1 inch from the mouth in a blowpipe flame. Drawing this out, we let it cool, and then filing the constricted portion we break it off and seal it in the flame. By shaking up and down vigorously for five to fifteen minutes, or preferably in a mechanical shaker, the bacteria are distributed evenly in the salt solution. A piece of platinum wire, twisted into corkscrew shape, and fused in the drawn-out end of the containing test tube helps in breaking up the bacterial emulsion and is a great aid in the preparation of streptococcal or diphtheroid vaccines.

The sealed test tube is then placed in a water bath at 60°C. and heated at this temperature for one hour. Again shake. The constricted sealed end is again filed off and a few drops shaken out in a watch glass for standardization, and at the same

time a few drops are deposited on an agar slant as a test for sterility. (Incubation for twenty-four to forty-eight hours should not show growth.)

Wright found that by taking a definite quantity of blood and the same quantity of bacterial emulsion, mixing them, then making a smear and staining, it was possible to determine the ratio of bacteria to red cells, and from this the number of bacteria per cubic centimeter could be determined. For example, if we find three bacteria to each red cell we should have 15,000,000 bacteria to 1 c.mm., there being 5,000,000 red cells to the cubic millimeter. As 1 cc. is 1000 times greater than 1 c.mm., there would be 15,000,000,000 bacteria in each cc. of such an emulsion, or vaccine, as it is termed.

The standardization made with a haemacytometer is best done by drawing up the vaccine to 0.5 with either the red or white pipette, according to concentration, and then sucking up one-twentieth of 1% dahlia in 1% formalin to 11 or 101. Allow the bacteria to settle on the shelf for ten minutes before counting. Count as in making a red count.

The use of a piece of amber glass in front of an incandescent light enables one to pick up the bacteria more satisfactorily as well as to differentiate bacteria from débris. A counting chamber with a depth of $\frac{1}{50}$ mm. is to be preferred to the ordinary $\frac{1}{10}$ mm. chamber as one can then begin the count after about five minutes' time for settling.

A satisfactory diluting fluid is that recommended by Callison. It is: Hydrochloric acid 2 cc., bichloride of mercury (1 to 500 aq. sol.) 100 cc., and sufficient 1% aqueous solution of acid fuchsin to color the diluting mixture a deep cherry red. The diluting fluid should then be filtered. The bichloride forms an albuminate on the surface of the bacteria which promotes rapid sedimentation and the fuchsin stains the bacteria.

Having determined the strength of the stock vaccine, we should prepare a dilute vaccine for injection.

This is most conveniently carried out by filling vials with 50 cc. of salt solution, plugging with cotton, then sterilizing in the autoclave. A sterile rubber cap is now drawn over the mouth of the vial. Sterility is insured by plunging the rubber cap and neck in boiling water. If the stock vaccine showed 5,000,000,000 bacteria per cc. and we desired to have a vaccine containing 200,000,000 bacteria per cc., it would be necessary to draw out 2 cc. of the salt solution by means of a sterile syringe needle inserted through the rubber cap and replace it with 2 cc. of the bacterial emulsion. Example: In introducing 2 cc. of a vaccine containing 5,000,000,000 bacteria per cc., we throw in 10,000,000,000 bacteria in a volume equal to 50 cc. Then each cc. of the 50 cc. in the bottle would contain 10,000,000,000 divided by 50, or 200,000,000 in each cc. If we only want a vaccine containing 100,000,000 per cc. we should add

only 1 cc. We now add 0.25% of trikresol to the vaccine in order to insure sterility. (Introduced with syringe, inserting needle through rubber cap.) When it is desired to remove a dose of vaccine, the neck and rubber cap of the bottle are dipped in boiling water, and the syringe is sterilized, if of glass, by boiling in water, or, if of metal, by drawing up vaseline or olive oil heated to 150°C. One can purchase ampoules which are sterilized and filled with a standardized emulsion. They are sealed in the flame and labelled with the bacterial content per cc.

The ordinary doses are: For vaccines of gonococci, streptococci, pneumococci, and *B. coli*, 5,000,000 to 50,000,000. For those of staphylococci 200,000,000 to 1,000,000,000.

Wilson gives the following minimum and maximum doses expressed in millions: *Streptococcus*, 6 and 68. *Gonococcus*, 45 and 900. *Meningococcus*, 300 and 900. *B. melitensis*, 700 and 1400. *B. coli*, 16 and 240. *B. typhosus* (treatment) 100 and 250. *B. typhosus* (prophylaxis) 500 and 1000. *B. pyocyaneus*, 34 and 1000. *B. pneumoniae*, 44. Staphylococci, 150 and 900. *B. tuberculosis* $\frac{1}{20,000}$ to 200 mg.

Smith, working at the Army Medical School with the typhoid-paratyphoid vaccine, claims to have found the Wright method subject to error. According to him, vaccines standardized by Wright's method to contain 1000 million bacilli per cc., actually contained 500 million per cc.

The Wright method of standardizing the official U.S. Army typhoid-paratyphoid vaccine was therefore replaced by the method originally described by Russell, Nichols and Stimmel. In this method standards are prepared cytometrically, using the Helber chamber. The vaccine suspensions are then compared nephelometrically with these standards. When the comparisons are made the ratios are read directly from standard curves which are easily plotted in the manner described by Dunham.

The finished product contains per cc. 500 million typhoid bacilli, 250 million paratyphoid "A" bacilli and 250 million paratyphoid "B" bacilli. It is felt that this present method of standardizing the vaccine is very accurate and makes for a satisfactory uniformity in the product.

Sensitized Vaccines.—These, prepared by treating the bacteria with the specific serum, cause less reaction than ordinary vaccines. To prepare them the bacterial growth is treated with its antiserum for three hours, thrown down in the centrifuge and the supernatant serum removed. After washing in salt solution they are emulsified in salt solution and killed at a temperature of 56°C. for one hour. Besredka has used living sensitized bacteria in typhoid.

The question of the best method of preparing vaccines for prophylactic use is still unsettled. The greatest difficulty has been experienced in making vaccines of the Shiga bacillus on account of the great toxicity of such preparations.

Thompson has recently carried out some very important experiments at the Lister Institute. He worked with vaccines, heated to 56°C. for one hour, prepared

by ordinary methods as well as with organisms sensitized by treatment with specific serum. In another series of vaccines he sterilized ordinary cultures as well as sensitized ones with 0.5% phenol in normal saline. He found that sterilization by heat not only destroyed much of the immunizing power of the vaccines, but that such vaccines, whether of ordinary bacterial emulsions or of sensitized organisms, showed great toxicity upon their being injected and the heated sensitized ones were somewhat more toxic than the nonsensitized organisms. Dean has used "eusol" for Shiga vaccines.

On the whole it would seem that sterilization with 0.5% carbolic or 0.25% trikresol, using ordinary bacterial emulsions, is better than other methods.

Of course living organisms subjected to their specific serum have been recommended in the case of typhoid but such methods are certainly not devoid of danger and are not to be recommended for the present.

Detoxication of Vaccines of Gram Negative Bacteria.—Wherry and Bowen recommend the method of Costa and Ramon. This method of preparation greatly reduces the toxicity of antigens prepared from members of the typhoid, paratyphoid, dysentery and coli group, and that of the gonococcus. Much larger doses of antigen may be used for prophylactic immunization, and for treatment.

With aseptic precautions the cultures are sedimented and resuspended in sterile 0.9 NaCl solution. To this is added sufficient 40% formalin to make a 1–100 dilution. The tube is corked tightly and incubated at 37 degrees C. for 24–48 hours. Sediment and wash three times with sterile 0.9% NaCl solution. Test for sterility. Preserve in 0.9 NaCl solution containing 0.5% carbolic acid.

Larson employes sodium ricinoleate for detoxication of streptococcus antigen used for active immunization of scarlet fever.

General Consideration as to Vaccine Therapy.—While there is indisputable evidence as to the value of bacterial vaccines in prophylaxis, as shown in typhoid prophylaxis, yet there is considerable evidence to minimize their value therapeutically, as shown by Leake. Whittington divided 230 cases of typhoid into two groups, one receiving vaccines, the other treated in the ordinary way. His results were as follows:

	Mortality, per cent.	Average days fever	Relapsed cases, per cent.	Cases with complications or sequelae, per cent.
Vaccine-treated cases.....	25	29.2	10.4	49.5
Controls.....	21	26.1	7.8	46.0

Park tested the value of pertussis vaccines in the treatment of a large number of cases of whooping cough, selecting alternate cases for the vaccine treatment. It was found that the specific treatment showed no superiority over nonspecific ones.

Intravenous injections of bacterial proteids, typhoid vaccines being usually employed, have been much advocated for chronic joint affections. It has been found very dangerous to exceed 0.3 cc. of the standard typhoid vaccine and we have noted

one fatal result, with anaphylactic death, at the time of the second intravenous injection of such a vaccine.

There is no question but that in many cases of furunculosis we obtain brilliant results with autogenous staphylococcus vaccines.

Hypersensitiveness

It may be stated (Zinsser) that hypersensitiveness exists whenever an individual reacts specifically to contact with a given substance with symptoms that fail to develop upon similar contact in a normal individual of the same species.

There is at the present time no agreement with regard to the terminology to be applied to the phenomena of hypersensitiveness, a fact to be expected in view of the meager knowledge we possess concerning the underlying causes of the phenomena and their mechanism.

As will have been noted, Coca and others make a sharp distinction between, on the one hand, *anaphylaxis*, in which an antigen-antibody mechanism is demonstrable, and, on the other hand, the group of reactions commonly termed *allergies* or *idiosyncrasies* in which no such mechanism has been detected.

Wells, on the contrary, believes that sufficient relationship obtains between the various phenomena of hypersensitiveness to permit grouping all manifestations of altered reactivity together under the term "allergy." Hanzlik also, while separating the phenomena of hypersusceptibility into two classes, anaphylactic and anaphylactoid, believes that, notwithstanding unexplained differences, both sets of phenomena rest on a common basis. Zinsser likewise asserts that the distinction between anaphylactic phenomena (protein anaphylaxis) and anaphylactoid (allergies, idiosyncrasies) is artificial, and useful only as a guide in investigation, he being convinced that all forms of hypersensitiveness depend on common fundamental biologic laws.

Anaphylaxis.—It has been stated by Muir and Ritchie that active research as to anaphylaxis may be dated from the discovery of the *phenomenon of Theobald Smith.* This, a guinea pig horse serum reaction, was discovered by Theobald Smith in his work with diphtheria antitoxin sera on guinea pigs. In the case of rabbits this phenomenon bears the name of Arthus. Various authors, notably Rosenau and Anderson, have confirmed Smith's observation that, if after a period of incubation of at least ten days following the primary injection of horse serum, a guinea pig is given a second one, there are produced symptoms of respiratory embarrassment and convulsions followed at times by death. It is assumed in such case that the primary injection has at the end of the requisite period of incubation "sensitized" the body cells to the particular alien proteid introduced. Extremely small

amounts of serum are sufficient to sensitize (0.0001 cc.), but for anaphylaxis production much larger amounts are required: 0.01 to 0.3 cc. when given intravenously or intracardially, and 1 or 2 cc. when given subcutaneously. The condition is transmissible from mother to offspring, probably through the placental blood. Desensitization may be effected by repeated injection of small doses of the specific antigen.

Anaphylactic shock.—The group of symptoms constituting the so called anaphylactic shock is apt to set in within a few minutes after the second injection and is often preceded by restlessness and great excitement. The dyspnoeic manifestations, such as coughing and rapid breathing, are accompanied with cardiac weakness and great fall of blood pressure. The more serious convulsive symptoms, followed at times by death in from a few minutes to one hour, are more apt to appear after intracerebral injections than after intraperitoneal.

Criteria of true anaphylaxis.—H. Gideon Wells in a review of the problems of anaphylaxis states that the following seven criteria must be complied with before the reaction can be called one of true anaphylaxis:

1. The observed toxicity of the injected material must depend upon the sensitization of the animal; i.e., the substance must not produce similar symptoms in nonsensitized animals.
2. The symptoms produced must be those characteristic of anaphylactic intoxication as observed in the usual reactions with typical soluble proteins, being therefore the same for all antigens with the same test animal, but differing characteristically with each species of animal.
3. It should be possible to demonstrate passive sensitization with the serum of sensitized animals.
4. It should be possible to demonstrate typical reactions in the virgin guinea pig uterus strip.
5. It should be possible to demonstrate amelioration or prevention of the bronchial spasm in guinea pigs by proper use of atropin and epinephrin.
6. The possibility that the observed symptoms are caused by capillary thrombosis or embolism must be excluded.
7. After recovery from anaphylactic shock there should be exhibited a condition of desensitization under proper conditions.

Pathological physiology.—Anaphylactic reactions have been produced in many species of mammals, but only in the guinea pig, rabbit and dog has the process been thoroughly studied. As between species, the phenomena are different; but they are always the same in any one species without relation to what antigen is being used. It is the peculiar susceptible condition of the subject, not the exciting substance, that determines the physiologic reaction when the specific contact occurs. In the guinea pig there is respiratory difficulty, caused by a spasm of the bronchial muscula-

ture. In the rabbit there are circulatory disturbances with fall in arterial blood pressure and marked distention of the right side of the heart. In the dog there is engorgement of liver and splanchnic circulation with subsequent fall of systemic blood pressure. Actual symptoms seem due chiefly to alterations in the reticulo-endothelial system which lead to capillary permeability.

Passive sensitization.—The general acceptance of the view that true anaphylaxis represents an antigen-antibody reaction is largely based on the phenomenon of passive sensitization. When serum of a sensitized animal is injected into a normal animal the latter also is renderd sensitive, but always after the lapse of a certain period of time, usually four hours. This passive anaphylactic sensitization seems to disappear in two weeks.

Nature of reaction.—It is not known how the manifestations of anaphylactic shock are produced. Various theories have been advanced. According to Vaughan and others the parenteral introduction (hypodermic as opposed to alimentary tract or enteral introduction) of foreign proteids excites the formation of specific ferments in the cells and fluids of the animal injected. After the ferment is formed a second injection of the same proteid activates the ferment which splits up the proteid into a poisonous and nonpoisonous portion, the former causing the symptoms of anaphylaxis, or of disease in the case of the poisonous split proteid of bacterial pathogens.

Although a point of distinction often made between anaphylaxis and allergy is that in the former specific circulating bodies are demonstrable, and the anaphylactic state transmissible by transfusions of blood, observations suggest that the union of antigen and antibody within the blood stream is relatively unimportant and that in both cases shock depends, at least largely, upon the union of antigen and antibody within or upon the surface of tissue cells. This is particularly true in the instance of the anaphylactoid phenomena in which the reaction may be conceived as depending on sessile cellular antibody elements.

Hypersensitiveness in Man. *Anaphylaxis in man.*—It is important to note that very rarely need there be concern over possible ill effects following the administration of a second injection of diphtheria antitoxin, even after the completion of the period of anaphylactic incubation. Serious reactions appear more frequently following the primary injection and occur then especially in asthmatics and in those who are already sensitive to horse emanations. Deaths have also been reported. In such cases, the patient within a few minutes after the single injection of serum becomes restless, shows symptoms of respiratory embarrassment and cyanosis and may be dead in 5 to 40 minutes.

Boughton reports the death of a man who was given 1 minim of horse serum intravenously to desensitize him for "horse asthma." This is the smallest fatal dose on record, the smallest amount causing death being usually 1 cc. At the autopsy of this case, there were found great distention and emphysema of the lungs, intense injection of abdominal veins and some subserous haemorrhages. The thymus was not enlarged. Microscopically the kidney showed the most marked changes, there

being intense passive hyperaemia with oedema of the epithelium of the convoluted tubules.

Desensitization.—Vaughan recommends that when a condition of this kind is to be feared one should attempt to *desensitize* the patient by giving only about 0.1 cc. of the serum at first. After an interval of two hours, provided no untoward symptoms set in, the full amount of the injection should be given. Besredka advises heating the serum to 56°C. as a guard against anaphylactic shock.

Where large doses of serum are to be given intravenously, as in type I pneumonias, a skin test should be made to see whether the patient is highly sensitive to horse serum. For this inject intradermally about 0.2 cc. of a 1:10 dilution of horse serum in saline. If sensitiveness exists a genuine urticarial wheal will develop in about five minutes to begin to fade away after an hour or so. Even where patients do not show sensitization, it is well, as a precaution, to give a subcutaneous injection of 1 cc. before giving the serum intravenously.

Clock states that the skin test for determining hypersensitiveness to horse serum is unreliable. Apparently 94 per cent. of patients who gave a positive skin test did not develop a systemic reaction when horse serum or antitoxin was injected. He favors an eye or conjunctiva test in which a 1:10 dilution of horse serum or antitoxin is instilled into the conjunctival sac. A severe conjunctival reaction indicates that a severe reaction may be expected if any amount of horse serum or antitoxin is injected. The eye reaction may be neutralized by the instillation of adrenal solution.

Where a patient shows marked sensitization, give very small doses of serum subcutaneously every half hour, commencing with 0.02 cc. and gradually going up to 1 cc. It may be well further to follow the subcutaneous desensitization with small doses, intravenously, at half-hour intervals.

It is also advised, when time permits, to ascertain if there exists a dangerous degree of hypersensitiveness by sensitizing guinea pigs passively with the serum of the patient and twenty-four hours later to inject them with the curative serum which it is proposed to employ. If untoward results occur in the guinea pigs the patient should not receive the injection.

Serum sickness.—In this an erythematous rash or urticaria associated with more or less oedema comes on after eight to twelve days from the time of the first and only injection of horse serum. Some cases show also joint pains. A subsequent injection of the same protein leads to the appearance of similar symptoms and signs, usually within 24 hours.

Hypersensitiveness to various pollens and certain foods.—Hay fever is due to the pollens of certain plants, the flowering period of these plants determining the seasonal prevalence of the disease. Persons subject to this condition suffer, after exposure, from urticarial and mucous membrane manifestations with cough and coryza and at times asthma. The same symptom-complex follows ingestion of certain foods (eggs, shell-fish) in susceptible individuals. Hypersensitiveness

to animal emanations also belongs to this group, a typical example being horse asthma.

The antigen-antibody reaction so apparent in true anaphylaxis does not explain these phenomena. In these conditions there are no antibodies free in the circulation. Coca, believing that the hypersensitiveness exists naturally and not as the result of artificial methods of immunization (parenteral injections), groups these conditions and serum sickness under one term, *allergy*, and eliminates them from the category of anaphylaxis reactions. He describes allergy as a natural inherited condition of hypersensitiveness, which affects only human beings and is not dependent in any way on immunological antibodies.

For the specific diagnosis and treatment of hay fever aqueous extracts of various pollens (ragweed, June grass, maize, timothy, red top, etc.) have been prepared. Persons who develop hay fever upon exposure to one or more of such pollens show skin reactions to those to the action of which they are susceptible. This reaction shows itself as a typical wheal which begins to develop in a few minutes and when well marked reaches the diameter of an inch or more. For diagnosis, the usual practice is to make a number of scratches on the arm at least two inches apart and then to apply a series of dilutions of such extracts to the scratches. At first we use the undiluted extract of the various pollens to determine the causative one. It having been determined, we then use dilutions of 25, 10, 5, 1%, or less, of the extract involved and note the dilution which fails to show a positive reaction. For treatment, about 5 drops of the dilution which fails to show a definite response is injected subcutaneously. At intervals of about three or four days we repeat the injections using increasing dosage. The subcutaneous injection should not be made until all manifestations of the skin test have subsided. This also holds true for subsequent subcutaneous injections.

Various other extracts also are on the market for the specific diagnosis of hypersensitiveness to such substances as maize, wheat, egg, etc.

It is important that those engaged in this type of work always keep at hand a hypodermic syringe containing adrenalin.

Idiosyncrasy.—This term is rather generally used in connection with abnormal response to drug administration, e.g., of quinine and acetyl salicylic acid. The reaction is similar to the one described for hay fever. A practical test for quinine idiosyncrasy is to apply a drop of 10% solution of quinine to a scratch on the arm and note the wheal formation. Methods for overcoming this hypersensitiveness by repeated injections of very small doses of the drug have been at times successfully employed.

Cutaneous hypersensitiveness.—While Coca includes all conditions manifested by cutaneous hypersensitivity in allergy and thus eliminates them from the domain of anaphylaxis, Kolmer believes that there are true anaphylactic skin reactions, caused by the interaction of a specific

anaphylactic antibody and specific anaphylactogen largely within or without the cells with the formation of a diffusible irritant, capable of producing acute hyperaemia, oedema and leukocytic infiltration of the skin.

Krause, however, finds no reason to consider hypersensitiveness necessarily related to anaphylaxis. He showed that one may sensitize guinea pigs to tuberculoprotein so that they will give typical systemic anaphylactic reactions, but will not show the tuberculin skin reaction. Skin sensitiveness can be obtained only by producing an actual tuberculous infection in the animal. A von Pirquet reaction may be obtained with a man having a focus of tuberculosis, but it is not obtained with a nontuberculous individual who has received tuberculin injections subcutaneously.

The immediate reaction in revaccination with cowpox virus was first noted by Jenner and later fully described by von Pirquet. Several recent writers have attempted to show that it is a distinct immune reaction. The reaction undoubtedly is due to some altered immunological state in the previously vaccinated individual and when it occurs at a secondary vaccination, a *known potent* virus being used, may be accepted as signifying that the individual is immune. The mechanism involved in the production of this reaction is not, however, fully understood.

It is characterized by the appearance within a few hours at the site of vaccination of an area of hyperaemia distinct from the traumatic reaction. Within 5 to 10 hours this area becomes distinctly palpable, the papule reaching its maximum of development in about 36 hours. At this time it is about 4–5 mm. broad, brownish-red in color, and is covered by a yellowish crust. The reaction disappears during the following week, but a slight pigmentation will, as a rule, remain for months.

The *Schick reaction* has no relation to what we ordinarily call hypersensitive skin reactions. The injection of diphtheria toxin in an individual who has insufficient diphtheria antitoxin in his circulation to neutralize the toxin is followed by a typical Schick reaction, which is probably due to the irritating action of the toxin at the site of injection. In case the individual has enough antitoxin, i.e., is immune, the toxin is neutralized and no reaction appears.

Bacteriophagy (D'Herelle)

Thwort, and later d'Herelle, observed that when Berkfeld filtrates of cultures of certain bacteria were added to cultures of other bacteria a solution of the micro-organisms resulted. The process of dissolution is termed bacteriophagy, and the agent causing the dissolution has become known as the bacteriophage.

The exact nature of the solvent agent is in dispute. It is assumed to be separable from the bacterial cell, and to be ultramicroscopic, inasmuch as it is transmissible in bacteria-free filtrates; but whether it is particulate, or of the nature of a catalyst or enzyme, has not been agreed upon.

Bordet and Ciuca believe that, as a result of artificial conditions imposed by culturing, there appear variant strains which display the property of autolysin production as an inheritable characteristic. Hadley similarly concludes that the phenomenon is to be conceived as one of transmissable bacterial autolysis, and that the development of this unusual property in certain bacterial strains is a manifestation of abnormal functioning of the reproductive system, he thus linking it with other dissociative phenomena and viewing it with them as an expression of microbic instability, in particular, a physiologic response of the strain to senility or to unfavorable environment, which may reach an extreme degree, then producing the striking "suicidal cultures."

Zinsser, however, influenced by work done in his laboratory by Kuttner and Callow, is inclined to attribute the lysis to the action of an enzyme, which, probably, is elaborated by all bacteria, but which is normally held in check by inhibitory substances. The phenomenon then, in his opinion, is primarily due to lack of inhibitory substances, or to their failure to act.

d'Herelle himself, who has been perhaps the most active investigator of the problem, is convinced that the active lytic agent is a parasite of bacteria which he has named *Protobios bacteriophagus* (syn. *Bacteriophagum intestinale.*) He also maintains that for certain diseases, notably bacillary dysentery, staphylococcus infections, and colon bacillus infections, "phagotherapy" is becoming established as a procedure of recognized therapeutic value.

PART II

STUDY OF THE BLOOD

CHAPTER XIII

THE TECHNIQUE OF CLINICAL BLOOD EXAMINATIONS

General Considerations

Many of the important clinical examinations of the blood are discussed in chapters dealing with special subjects of internal medicine, such as agglutination and complement fixation reactions under "Immunity" (p. 234), blood chemistry and examinations for occult blood in the chemical sections of the "Appendix" (p. 718), blood examinations for protozoa under "Animal Parasitology" (p. 389), and blood culturing methods in the chapter on the bacteriology of the blood in Part IV (p. 618).

In this chapter are treated the methods belonging more strictly to the clinical microscopy of the blood, together with the technique of blood grouping. When blood is desired for culturing and performing serum reactions, the specimen is usually obtained by venepuncture with a hypodermic needle; while in those examinations dealt with in this chapter, such as haemoglobin determinations, blood cell counting and making smears for a differential count, it suffices to obtain the blood from a skin puncture.

Routine blood examinations.—The two examinations which may be considered routine ones to be made on all patients entering a hospital are the haemoglobin determination and the leukocyte count. If the leukocyte count should be approximately 10,000, or above, a differential count should follow; and when the haemoglobin percentage is below 80, a red count is indicated.

In this connection it is very desirable that the laboratory worker be acquainted with the presumptive diagnosis of the case, as the limitations of laboratory findings are better understood by him than by the clinician. While a nonmedical

laboratory worker may have technical skill of a high order it is impossible for him properly to interpret the significance of his results and it cannot be too strongly insisted upon that the best interests of the patient can only be served when the laboratorian is capable of studying the clinical as well as the laboratory findings.

Some clinicians, as a routine, call for examinations which involve many of the procedures of clinical microscopy of the blood. It is impossible to give the same care and thought to such a succession of examinations as would be devoted to one specially requested. If the laboratorian were not asked particularly to search for malarial parasites he might well overlook a rare parasite in the stained smear of such an extensive routine examination.

Blood Preparations

To obtain blood, except for blood cultures or serum reactions, use either a platinoiridium hypodermic needle which can be sterilized in the flame, a small lancet, or a Hagedorn surgical needle with cutting edge. A surgical needle may be handled conveniently, and kept under antiseptic conditions, by inserting the blunt end in the cork of a small vial filled with alcohol. When the needle is removed for use, the adhering film of alcohol is ignited.

Puncturing the Skin.—When using such surgical needles it is a good plan to sharpen the cutting edge on a fine-grained whetstone. Afterward the needle should be sterilized by boiling. Sterilization of a needle in the flame blunts the cutting edge. A steel pen with one nib broken off or the glass needle of Wright may also be used. To make a glass needle, pull straight apart a piece of capillary tubing in a very small flame. Tap the fine point to break off the very delicate extremity. Scarcely any pain attends the use of such a needle. Before puncturing, make the finger hyperaemic by dipping in hot water and rubbing with gauze. In puncturing either the tip of the finger or lobule of the ear, a quick piano-touch-like stroke should be used. The ear is preferable, as it is less sensitive and there is less danger of infection. For little children the big toe or heel is more satisfactory. Before puncturing, the skin should be cleaned with 70% alcohol and allowed to dry.

Note that in order to secure in the specimen a cell count that corresponds to that obtaining in the circulation as a whole it is necessary to massage the ear vigorously prior to making the puncture. Subsequently there should be no manipulation of the part, the blood examined being that which exudes freely. This procedure renders more likely the finding of blood parasites.

The first drop of blood which exudes should be taken for haemoglobin estimation, subsequent ones being used for the blood pipettes and smears. If it is necessary to make a complete blood examination, the puncture should be sufficiently deep to insure a free flow of blood without undue squeezing.

Haemoglobin Estimation

The following summary of the relative values of the various haemoglobinometers in estimating the haemoglobin content of the blood was recently published in the Journal of the American Medical Association.

When one stops to realize that nearly all haemoglobinometers, which record the normal as 100 per cent, have a different haemoglobin equivalent and, further, that men, women and children have a different normal, it is apparent that considerable confusion would be avoided if all haemoglobin estimations were recorded in grams per hundred cubic centimeters or actual percentage, as is the case with other blood determinations. All instruments, if properly calibrated, would then yield the same results.

The following haemoglobinometers have apparently been calibrated on the basis of 100 per cent being equivalent to the grams of haemoglobin given below:

	Gm. per 100 cc.
Dare	13.77
Haldane	13.8
Oliver	15.0
Von Fleischl-Miescher	15.8
Tallqvist	15.8
Sahli	17.2

The von Fleischl-Miescher is the most accurate of the older clinical instruments, and has been employed as a standard of reference for many instruments.

Haldane, who modified the older Hoppe-Seyler carboxy-haemoglobin method, took an oxygen capacity of 18.5 per cent as his standard for men (equivalent to 13.8 per cent of haemoglobin). He states that with women he found an average oxygen capacity of 16.5, and with children 16.1 per cent, thus necessitating a different standard of 100 for women and children.

Williamson made a study of the haemoglobin content of nearly a thousand normal persons in Chicago, employing the accurate spectrophotometric method of Hüfner. For the adult male he found an average of 16.92 Gm. of haemoglobin per hundred cubic centimeters of blood, and for the adult female, 15.53 Gm. In children, the figures varied roughly from 13 Gm. of haemoglobin at 6 years of age to 15 Gm. at 15 years.

Employing the Van Slyke method for oxygen capacity, Hayden obtained an average haemoglobin of 15.83 Gm. on twenty males between 18 and 30 years of age, and 15.23 Gm. on twenty men between 30 and 50, and 13.34 Gm. on twelve women between 20 and 40. Recalculating his figures on the basis of 5 million red cells, he obtained an oxygen capacity of 20.85, equivalent to 15.57 Gm. of haemoglobin. He suggests that all haemoglobinometers should be calibrated with the Van Slyke apparatus on this basis, so that 100 per cent equals 15.6 Gm. per hundred cubic centimeters of blood.

Newcomer has modified the Sahli acid haematin method by substituting for the standard solution a colored glass disc which may be used with any colorimeter made

on the Duboscq pattern. Employing the Van Slyke oxygen capacity method, Myers recalibrated one of these discs and found it accurate to 1 per cent.

In the Newcomer haemoglobinometer the normal of 100% is equivalent to 16.92 Gm. of haemoglobin per hundred cubic centimeters found by Williamson to be normal for adult men.

Newcomer's Method.—The standard disc is placed in one of the light paths of the colorimeter either above or below one of the cups which is filled with water. Exactly 20 cubic millimeters of blood from a skin puncture is placed in 5 cubic centimeters of N/10 HCl (the pipette of the Sahli haemoglobinometer may be used), and mixed well. This gives a dilution of 1 in 251. Adjust the plunger until the two fields match and make the reading. The amount of haemoglobin in grams per 100 cc. is calculated by the aid of the following table which takes into account two variable factors: (1) the time which has elapsed since the blood dilution was made, and (2) the thickness of the colored glass disc.

NEWCOMER'S TABLE FOR USE WITH THE STANDARD DISC

To Obtain Grams Haemoglobin per 100 cc. Blood Divide the Colorimetric Reading into the Appropriate Figure

Minutes since dilution	Thickness of the colored glass in millimeters										
	0.95	0.96	0.97	0.98	0.99	1.00	1.01	1.02	1.03	1.04	1.05
10	94.4	95.4	96.4	97.4	98.4	99.4	100.4	101.4	102.4	103.4	104.4
15	93.1	94.1	95.0	96.0	97.0	98.0	99.0	100.0	101.0	102.0	103.0
20	92.5	93.5	94.5	95.4	96.4	97.4	98.4	99.4	100.4	101.3	102.3
30	91.8	92.8	93.8	94.8	95.7	96.7	97.7	98.6	99.6	100.5	101.5
40	91.6	92.5	93.5	94.5	95.4	96.4	97.4	98.3	99.3	100.3	101.2
Final	90.6	91.6	92.5	93.5	94.4	95.4	96.4	97.3	98.3	99.2	100.2

Dare's Haemoglobinometer.—The Dare apparatus for the estimation of haemoglobin is very convenient and sufficiently accurate for clinical estimation. Its operation consists in the comparison of the patient's blood with a tinted glass wedge mounted on the revolving disc, both being observed through a tube that cuts off outside light. A drop of whole blood is taken up between two plane glass surfaces which act as a capillary pipette. This, carrying the blood, is inserted beside the tinted wedge, and comparison is made between the whole blood and the color scale. Illumination is obtained from a candle attached to the apparatus or from a small bulb and electric battery in the case of the newer models. The percentage of haemoglobin is then read directly from a scale attached to the disc carrying the colored wedge.

Sahli's Haemometer.—A simple and apparently very scientific instrument is the Sahli modification of the Gower haemoglobinometer. Instead of the tinted glass, or gelatin colored with picrocarmine to

resemble a definite blood dilution, Sahli uses as a standard the same coloring matter as is present in the tube containing the blood. By acting on blood with 10 times its volume of N/10 HCl, haematin hydrochlorate is produced, which gives a brownish-yellow color. In the standard tube, which is sealed, a dilution representing 1% of normal blood is used.

To apply this test, pour in N/10 HCl to the mark 10 on the scale of the graduated tube. Add to this 20 cu. mm. of the blood to be examined, drawn up by the capillary pipette provided. So soon as the mixture assumes a clear dark-brown

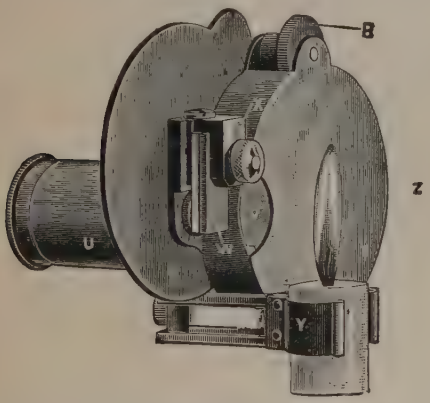


FIG. 67.—Haemoglobinometer of Dare: R, milled wheel; S, case inclosing the color disc; T, movable wing, which is swung outward; U, telescoping camera; V, aperture admitting light; W, capillary blood pipette; Y, detachable candle holder; Z, slot through which the precentage of haemoglobin is read.

FIG. 68.—Sahli's haemoglobinometer. (*Greene.*)

color, add water drop by drop until the color of the tubes matches. The reading of the height of the aqueous dilution on the scale gives the Hb. reading. The tubes are encased in a vulcanite frame with rectangular apertures. This gives the same optical impression as would plano-parallel glass sides.

The most accurate readings are obtained with artificial light in a dark room but almost as satisfactory comparisons can be obtained with natural light from a window. It is advisable to turn the ruled side around so that one may match colors without being influenced in his determination by the scale.

The apparatus must be kept in a dark place as strong light will change the color of the standard tube. It is recommended that the N/10 HCl be preserved with chloroform.

Tallqvist's Haemoglobin Scale.—This is a small book of specially prepared filter paper with a color-scale plate of 10 shades of blood colors. These are so tinted as to match blood taken up on a piece of the filter paper and are graded from 10 to 100. So soon as the blood on the filter paper has lost its humid gloss, the comparison should be made.

This may be done by shifting the blood-stained piece of filter paper suddenly from one to the other of the holes cut in each shade—the piece of filter paper being underneath the color plate; it is better, however, to match the colors with the blood spot against the scale rather than behind a preparation. Grawitz prefers to cut the stained spot from the filter paper and place it directly on the color scale.

At least a square centimeter of the filter paper should be stained by the blood. Daylight coming from a window at the rear or at the side should be used in making the comparison. When the Tallqvist scale is compared with the Newcomer method, it is found to give too low results on blood with a higher content of haemoglobin, and much too high results in cases of severe anemia. If the colored plate is not kept in the dark, the tints tend to fade.

To Count Blood Corpuscles

Haemacytometer.—The instrument almost universally used is the Thoma-Zeiss haemacytometer. The apparatus consists of two pipettes, one for leukocytes, graduated to give a dilution of 1 to 10 or greater; the other for red cells to give a dilution of 1 to 100 or greater. The white pipette has the mark 11 above the bulb and the red pipette the mark 101. In addition, there is a counting chamber.

Counting chamber.—The Thoma, or closed type of cell, with Thoma, Türck or Zappert ruling, is now little used. The parallel trench form of chamber, first suggested by Alferow and Bürker has been almost universally replaced by the Levy Counting Chamber with Neubauer ruling made in one-piece solid construction with the ruling made directly on the glass slide, which obviates any possibility of separation between the slide and the ruled glass strip.

The outstanding advantage of the parallel trench chamber is that it permits the introduction of the diluted blood into the space between the ruled area and the cover glass by capillary attraction after the cover glass is correctly adjusted to the slide which, as Bürker definitely proved, insures a more uniform distribution of the corpuscles than is possible with the old circular or closed Thoma form of construction.

The double chamber, also suggested by Bürker, with two rulings, one on either side of a transverse central trench and called by some writers the "H-trench," now

seems destined to replace single ruled chambers because of the convenience with which two counts can be made at the same time without the necessity of cleaning and refilling.

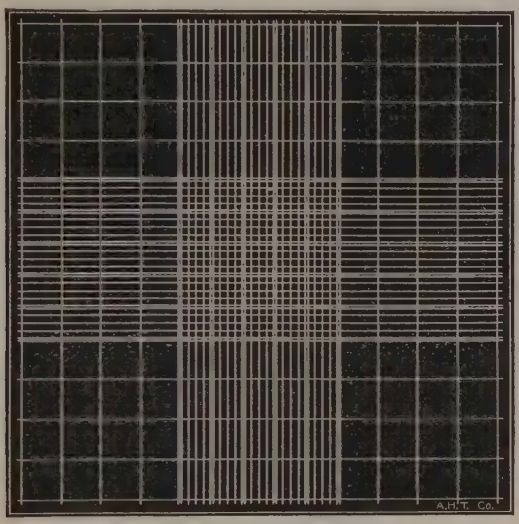


FIG. 69.—Entire area of improved Neubauer ruling showing split boundary lines. (400 small squares available for counting.)

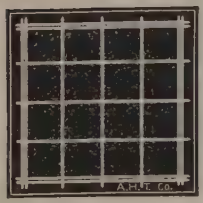


FIG. 70.—Group of 16 smallest squares of improved Neubauer ruling showing split boundary lines. Entire group visible simultaneously with 4 mm. objective.

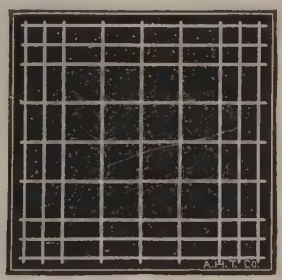


FIG. 71.—Group of 16 smallest squares of original Neubauer ruling showing boundary by fifth squares with extra line in middle of each. The border squares necessitate readjustment of focus.

Improved Neubauer Ruling.—In the improved Neubauer ruling the central square millimeter is divided—as in the Thoma and in the original Neubauer—into 400 small squares each of $\frac{1}{400}$th square millimeter area. The improvement consists in the division of these small squares into twenty-five groups of sixteen each by a

new "split" fifth line. This appears under the microscope as a transparent boundary for each group of sixteen small squares.

In the Thoma and in the original Neubauer rulings this was accomplished by an extra line in the middle of every fifth square.

Levy-Hausser Counting Chamber.—The ruled glass slide made of one solid piece of glass is carried in a moulded Bakelite holder of convenient size and shape for use on the microscope stage, which protects the glass slide against scratching of the under side and against breakage.

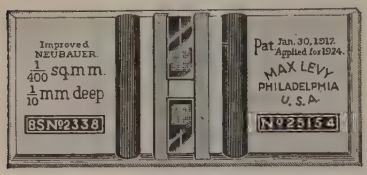


FIG. 72.—Levy counting chamber.

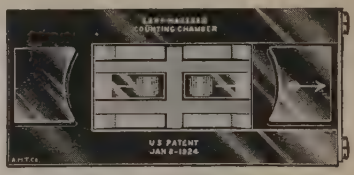


FIG. 73.—Levy-Hausser counting chamber.

The ruled glass slide can be readily removed from the Bakelite holder by loosening of the metallic clamp and withdrawal of the protecting slide, although this is not necessary, either for the cleaning of the ruled glass slide or for the identification of the ruling. This interchangeability provides for the use of ruled glass slides with improved Neubauer ruling, either single or double, and Fuchs-Rosenthal and Helber cells in the same holder.

FIG. 74.—Haemacytometers with pipette closures and complete clinical outfit.

Trenner diluting pipettes.—Unavoidable errors in the count occur with the original Thoma diluting pipettes because of the inability of the operator to stop the flow of blood exactly at the selected graduation on the capillary stem. The Trenner pipette utilizes surface tension in filled tubes of small bore, i.e., capillarity, for precise adjustment of capillary volume and thereby provides extremely accurate control of dilution.

The original Thoma diluting pipette now in general use is made by blowing a bulb in a capillary tube, which procedure causes the capillary to widen or flare into a

funnel shape at the point of its junction with the bulb, as shown in Fig. 76. The Trenner diluting pipette is made by fusing to a separately made bulb a piece of straight capillary tubing, the upper end of which terminates abruptly in a ground and polished surface at right angles to the longitudinal axis, as shown in Fig. 78.

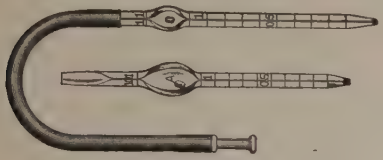


FIG. 75.—Thoma diluting pipettes.

FIG. 76.—Thoma diluting pipette bulb enlarged.

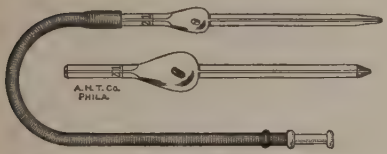


FIG. 77.—Trenner automatic diluting pipettes.

FIG. 78.—Trenner diluting pipette bulb enlarged.

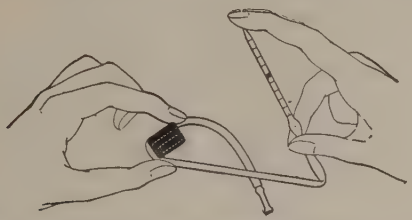


FIG. 79.—Closure for blood diluting pipettes showing (1) Closure in position on rubber tubing. (2) filled pipette closed with finger tip, and (3) rubber tubing sharply kinked prior to rotating tip into position in closure.

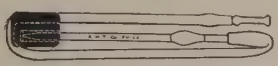


FIG. 80.—Closure for blood diluting pipettes showing pipette tip in position in closure. Pipette is now automatically sealed.

The capillary volume of this piece of tubing is adjusted to be exactly $\frac{1}{20}$th of the total volume content of the bulb of the white pipette and $\frac{1}{200}$th of the content of the bulb of the red pipette, as practically all blood counts are now made at these dilutions. This dispenses with the necessity for any engraving on the capillary tube since in use it is filled completely and there is need for only one graduation, i.e., that on the upper capillary, by which the dilution and total content of the pipette is controlled.

The bulbs are made somewhat larger than those hitherto supplied to insure increased accuracy in dilution.

Closure for haemacytometer pipettes, of hard rubber, or metal are obtainable for either permanent or temporary attachment to the rubber tubing. When the closure is in position on the rubber tube, the bore of the tubing is only slightly constricted so that suction is not interfered with during the filling of the pipette.

In addition to the admitted advantages of a satisfactory closure, this device further aids in both the convenience and efficiency of shaking the pipettes.

Red Cell Counts. *To make a red count.*—Having a fairly large drop of blood, apply the tip of the 101 pipette to it and, holding the pipette horizontally, carefully and slowly draw up with suction on the rubber tube a column of blood to exactly 0.5 or, in cases of severe anaemia, to 1. The variation of $\frac{1}{25}$ inch from the mark would make a difference of almost 3%. If the column goes above 0.5, it can be gently tapped down on a piece of filter paper until the 0.5 line is cut. Now insert the tip of the pipette into some diluting fluid and, revolving the pipette on its long axis while filling it, continue suction until the mark 101 is reached. A variation of $\frac{1}{25}$ inch at this mark would give an error of only about one-thirtieth of 1%. After mixing thoroughly by shaking from one to two minutes, the fluid in the pipette below the bulb is expelled, this being only diluting fluid. A drop of the diluted blood of a size just sufficient to cover the ruled area is allowed to flow under the cover glass of the counting chamber by capillarity. The solution must not flow into the moats on either side, nor should any bubble form under the cover glass.

After allowing three minutes for settling, the evenness of the distribution is determined either by microscopic examination with a 16 mm. objective, or by placing the slide upon the stage of the microscope and illuminating it with substage diaphragm wide open. If, with the slide in this position, the counting surface is viewed obliquely with the unaided eye, any irregularity in the distribution of the corpuscles is easily recognized by variations in the density of the blood film. If such variations are apparent, the cover glass should be removed, the chamber carefully cleaned and the procedure repeated until an even distribution is evident.

Diluting fluids.—Among diluting fluids Toisson's is probably the best known:

Sodium chloride	1 gram
Sodium sulphate	8 grams
Glycerin	30 cc.
Distilled water	160 cc.

Dissolve the sodium chloride and the sodium sulphate in the glycerin-water and add sufficient methyl or gentian violet to give a rich violet tint.

Hayem's solution is very satisfactory and is preferred by many workers. It has the following composition: Corrosive sublimate 0.5 gram, sod. chloride 1 gram, sod. sulphate 5 grams, 200 cc. of distilled water.

A 2.5% solution of potassium bichromate makes a very satisfactory diluting fluid in the counting of red cells. Recent samples have however haemolyzed red cells.

A salt solution of about 1% strength, tinged with about 1 drop of a saturated alcoholic solution of gentian violet to about 50 cc., is a good substitute, or the salt

solution alone will answer when no white count is to be made at the same time as the red one.

Method of counting.—It will be remembered that the small squares are $\frac{1}{20}$ mm. square. The depth of fluid from upper surface of shelf to lower surface of cover glass is $\frac{1}{10}$ mm. Hence each space embraced by the small square and the depth of fluid is $\frac{1}{4000}$ of the unit used in estimating number of corpuscles in blood, or 1 cu. mm. ($\frac{1}{20} \times \frac{1}{20} \times \frac{1}{10} = \frac{1}{4000}$). Count 100 of the small squares (this enables one to use decimals). Using the improved Neubauer ruling we note that in the central square millimeter there are 25 squares between double-ruled lines, each containing 16 small squares. Count the number of corpuscles in the 16 small squares contained in upper left-hand double-ruled square. Put down this count. Next count corpuscles in the adjoining 16 squares. Put down this count. Then in third 16 squares. Put down the number. Now move down to the next row of five double-ruled squares. Count the number of corpuscles in each of three 16-square spaces and set down the numbers for addition. We have now counted 96 small squares (6×16). Count at any place four additional small squares and add number of blood cells contained therein to those in the 96 small squares already counted. Divide the sum by 100, or simply point off two decimals. This gives the average for each small square. Multiply this by the dilution and then (as the small square represents only $\frac{1}{4000}$ cu. mm.) by 4000. This will give the number of corpuscles in 1 cu. mm. Example: 100 small squares contained 655 red cells. Pointing off, 6.55 equals average number of red cells per small square. Multiply by dilution (200) and then by 40000. $6.55 \times 200 \times 4000 = 5{,}240{,}000$.

At least 100 small squares, and preferably 200, should be counted. If the blood appears normal, one may simply count the number of red cells in five of the 16 small square spaces (80 small squares). Having added the numbers and multiplying by 10,000, you obtain the number of cells in 1 cu. mm. (Eighty small squares is $\frac{1}{50}$ of the unit of 1 cu. mm., or 4000 small squares. The blood dilution being 1 to 200, we have 10,000 [$= 50 \times 200$] $\times$ number of cells in 80 small squares.)

In counting, count corpuscles lying on the lines above and to the right. Do not count those lying on lines below and to the left. In the small squares count only corpuscles lying in the space or cutting the upper line. This prevents counting the same cell twice.

Reticulated Cell Count.—Take up blood in a white pipette to the 0.5 mark, and fill to the 11 mark with a diluent made by adding 2 drops of 2% potassium oxalate solution and one drop of sat. aqueous cresyl blue to 5 cc. of normal saline. After shaking the pipette for 3 or 4 minutes, mount on a slide, sealing with petrolatum. Count the number of reticulated cells occurring among 500 erythrocytes.

White Cell Counts.—Draw up the blood in the white pipette to the mark 0.5. Then, still holding the pipette as nearly horizontal as

possible, because the column of blood tends to fall down in the larger bore, draw up by suction, to the point 11, a diluting fluid which will disintegrate the red cells without injuring the whites. We then have a dilution of 1 to 20.

The best fluid is 0.5% of glacial acetic acid in water. This makes the white cells stand out as highly refractile bodies. Some prefer to tinge the fluid with gentian violet. The 0.5 mark is preferred because it takes a very large drop of blood to fill the tube up to the I mark and if there is much of a leukocytosis a 1 to 10 dilution is not sufficient. In leukaemic blood it is better to use the red pipette with the 0.5% acetic acid solution.

Making a preparation, exactly as was done in the case of the red count, we count all of the white cells in one of the large squares (1 sq. mm.). The cross ruling greatly facilitates this. Note the number. Then count a second and a third large square. Strike an average and multiply this by 10, as the depth of the fluid gives a content equal to only $\frac{1}{10}$ cu. mm. Then multiply by the dilution. Example: First large square 50; second large square 70; third large square 60. Average 60. Then $60 \times 10 \times 20 = 12{,}000$, the number of leukocytes in 1 cu. mm. of blood. A variation in the method of counting, which yields a truer average and simplifies calculations, is to add the counts from four large squares and multiply the sum by 50 [= 20 (for dilution) × 10 (for depth of cell) ÷ 4 (for number of squares counted)]. The count may be made with a low power ($\frac{2}{3}$-inch objective) as the leukocytes stand out like pearls. It is more accurate, however, to use a higher power, so that pieces of foreign material may be recognized and not enumerated as white cells. If one will accustom himself to comparing the distribution of the leukocytes in a well made, stained dried blood film, prepared according to Ehrlich's cover glass method, with that in a haemacytometer preparation, he can readily acquire an experience which will enable him to determine with considerable accuracy the degree of leukocytosis by the examination of a stained cover glass preparation alone. Furthermore, one can identify the leukocytes in a Giemsa-stained smear with the $\frac{2}{3}$-inch objective. This is specially true of the large mononuclears and transitionals the increase of which has such significance in the tropics.

Special diaphragms for the ocular, with a square opening just covering one of the large squares of the haemacytometer (400 small squares), may be purchased. A substitute may be constructed as follows: With the ocular micrometer in situ, note the number of its spaces which cover one side of a large square (1 mm.) in the microscopic field. Removing the ocular micrometer, the number of spaces determined are used to measure the sides of a square to be cut out of a piece of stiff paper. When this paper diaphragm is placed in the ocular, resting on the micrometer supports, the extent of field visible will be exactly 1 sq. mm. With such an opening one can count the leukocytes in unruled areas equal to a large square, where there is no ruling other than the central square (Thoma-Zeiss).

Combined Red and White Counts.—In the absence of a white pipette or when it is desired to make a white count with the same preparation as is used for the red one, especially if the ruling is of the old style (only central ruling and not in nine large squares as with Zappert and Türck), it is advisable to make use of the method

of counting by fields. With a Leitz No. 4 ocular and a No. 6 objective, with a tube length of 120 mm., it will be observed that the field so obtained has a diameter coinciding with a line formed by the sides of 8 small squares of the haemacytometer ruling, the radius of the circular field then being 4 × the side of a small square (0.05 mm.) or 0.2 mm. The total area then included in the circular field (πr^2) is $3.1416 \times (0.2)^2$ or 0.125664 square mm., which very closely approximates 50 times the area of a small square ($\frac{1}{400}$ square mm.). Hence each field, with the microscope adjusted as stated, contains 50 of the small squares, or $\frac{1}{80}$ of the unit of 1 cu. mm. of the diluted blood.

A single red cell lying at the circumference may be selected and used as a guide in moving the mechanical stage to obtain a new field. Example: As 2000 small squares would ordinarily be a sufficient number to count for a white count, this would require us to count the number of leukocytes in 40 of the designated microscopic fields (this, of course, is only one-half the unit, hence we should multiply by 2). Counted 40 fields and noted 50 white cells: 50 × 2 × 200 (the dilution in red pipette) = 20,000. Consequently 20,000 would represent the number of leukocytes in 1 cu. mm. of the blood examined.

Cleaning Apparatus.—After making a blood count, the haemacytometer slide should be cleaned with soap and water and then rubbed dry, preferably with an old piece of linen. The pipettes should be cleaned by inserting the ends into the tube from a vacuum pump, as a Chapman pump. First draw water or 1% sod. carbonate solution through the pipette, then alcohol, then ether, and finally allow air to pass through to dry the interior. If the interior is stained, use 1% HCl in alcohol. If a vacuum pump is not at hand, a bicycle pump or suction by mouth will answer.

Preparations for the Study of Fresh Blood

Many authorities prefer a fresh blood specimen to a stained dried smear in the study of parasites of the blood. In malaria in particular there is so much information as to species to be obtained from a fresh specimen that the employment of this method should never be neglected. While waiting for the film to stain one has five or six minutes which could not better be spent than in examining the fresh specimen which only requires a moment to make.

Manson's Method.—Have a perfectly clean cover glass and slide. Touch the apex of the exuding drop of blood with the cover glass and drop it on the center of the slide. The blood flows out in a film which exhibits an "empty zone" in the center. Surrounding this we have the "zone of scattered corpuscles," next the "single-layer zone" and the "zone of rouleaux" at the periphery. It is well to ring the preparation with vaseline. When desiring to demonstrate the flagellated bodies in malaria, it is well to breathe on the cover glass just prior to touching the drop of blood.

The method of Ross is very easy of application and gives most satisfactory preparations. Take a perfectly clean slide, and make a vaseline ring or square of the size of the cover glass. Then, having taken up the blood on the cover glass,

drop it so that its margin rests on the vaseline ring. By gently pressing down the cover glass on the vaseline, beautiful preparations are made which keep for a very long time. If it is desired to study the action of stains on living cells, this method is also applicable A very practical way to do this is to tinge o 85% salt solution containing 1% sodium citrate (the same as is used in opsonic work) with methylene azur, gentian violet, or methyl green. With a Wright bulb pipette, take up 1 part of blood, then 1 part of tinted salt solution. Mix them quickly on a slide and then deposit a small drop of the mixture in the center of the vaseline ring and immediately apply a cover glass and press down the margins as before. This method will be found of great practical value.

Vital Staining.—The stain generally used is Pappenheim's pyronine methyl green one. Neutral red, methylene azur or brilliant cresyl blue are also satisfactory. We prefer methylene azur. A particle of the stain is dissolved in about 1 cc. of 1% sodium oxalate in normal salt solution and to this is added a few drops of the blood to be examined. The mixture is well shaken and allowed to stand 10 minutes. Take up some of the sediment with a rubber bulb pipette and mount.

H. C. Ross describes a method using agar jelly tinted with cresyl blue. The agar is melted, poured on a slide and allowed to cool. A cover slip, on which has been deposited a drop of blood, is applied face downward on the agar. Reticulated cells are well stained, and leukocytic granules are plainly visible.

Preparation of Dried Films

When preparations are desired for a differential count, Ehrlich's method of making films is to be preferred, as the different types of leukocytes are more evenly distributed. In making smears by spreading, there is a tendency for the polymorphonuclears to be concentrated at the margin while lymphocytes remain in the central part of the film. Napier states that the most uniform results are obtained by making smears that cover small areas and counting every cell.

Cover Slip Films.—In *Ehrlich's method* we use perfectly clean dry cover slips. Take up a small drop of blood, without touching the surface of the ear or finger, on one slip and drop this immediately on a second one; as soon as the blood runs out in a film, draw the two cover slips apart in a plane parallel to their surface. Ehrlich used forceps to hold the cover glasses so as to avoid moisture from the fingers, but I find I can work more quickly and satisfactorily with the fingers alone. The method shown in Fig. 81 is a very convenient one.

For removing dirt and grease from the skin, preparatory to making blood smears, a mixture of 40 parts of acetone and 60 parts of alcohol is the best and quickest means. A bottle of this may be kept on hand, with the puncturing needle fixed in the stopper. Soap and water are more efficient than alcohol.

Slides and spreaders should be absolutely clean and grease-free. Scrubbing with soap and water, thorough rinsing and drying, then subjecting the slide to the flame to make it grease-free is satisfactory, but for cleaning a slide, nothing equals Bon Ami. Rub up some with the wet finger, rub the slide with the lather until there

is a friction squeak; let dry; polish with a clean, dry cloth. This is far better than soap and water, alcohol, ether and flaming combined. Note how a drop of water spreads on a glass so treated.

Smears on Slides.—Of the various methods of spreading films on slides, that described by Daniels is one of the best. In this the drop of blood is drawn along and not pushed along. The films are even, can be made of any desired thickness by changing the angle at which

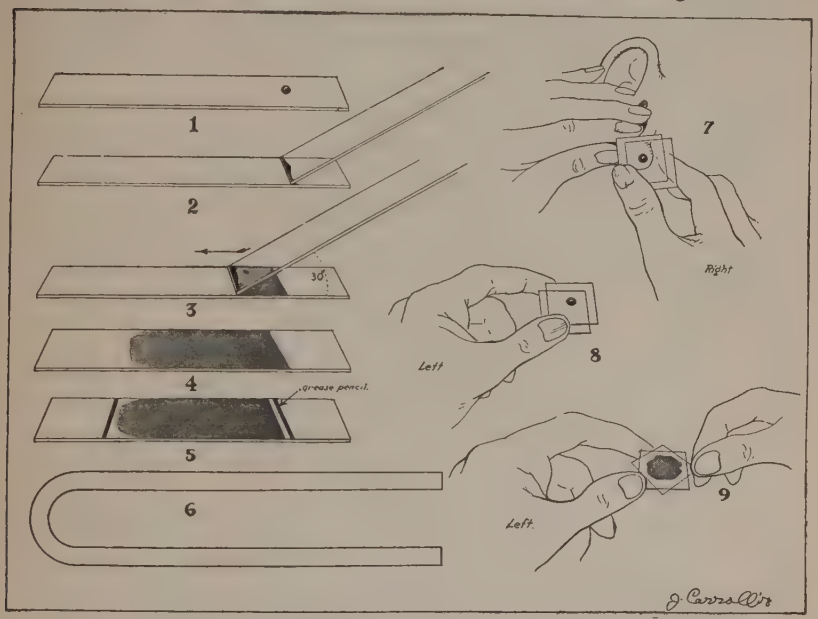


FIG. 81.—1, 2, 3, 4. Making blood smears on slide. 5. Smear ready for staining—grease marks prevent Wright stain from running over slide. 6. U-shaped glass tubing to hold slide in staining. 7. Right hand holding two cover glasses. One cover glass is being touched to drop of blood from ear. 8. Cover glasses transferred to left hand in preparing to place one cover glass on another and spread film. 9. Separating cover glasses by sliding one from the other.

the spreader-slide is held, and there is little liability of crushing pathological cells. Touch the end of a clean slide, to be used as a spreader, to a drop of blood. Holding this slide at an angle of 45°, bring the drop into contact with a horizontal slide near one end, and, as soon as the blood runs out along the line of contact, advance the spreader-slide with an even sweep to the other end. The blood is pulled or drawn behind the edge of the advancing slide. An angle less than 45° makes a thinner film; one greater, a thicker film.

Instead of a slide a square cover glass may be used and if the edge be smooth it makes a more satisfactory spreader than the slide.

Instead of the Daniels method I prefer to take up the drop of blood on the slide on which the smear is to be made, about ½ inch from the end. Then apply the spreader-slide and so soon as the drop runs along the end of the spreader-slide proceed as above described. This method is shown in Fig. 8.

Spreaders.—Of the various methods of making smears by means of cigarette paper, rubber tissue, needles, etc., the best seems to be to take a piece of capillary glass tubing and use this instead of a needle in making the film. There is one advantage about the strip of cigarette paper touched to the drop of blood and drawn out along the slide or cover glass, and that is that it is almost impossible not to make a working preparation by this method.

Thick Film Methods.—Such methods are of the greatest practical assistance in searching for malarial parasites, when in very small numbers in the peripheral circulation, trypanosomes, relapsing fever spirochaetes and filarial embryos. Ruge's method so brings out the polymorphonuclears that such a technique can be used also for the determination of the opsonic index.

Method of Ross.—In this about one-half of a drop of blood is smeared out over a surface about equal to that of a square cover glass and allowed to dry. It is then flooded with a 0.1% aqueous solution of eosin for about fifteen minutes. The preparation is then gently washed with water and then treated with a polychrome methylene blue solution. After a few seconds this is carefully washed off and the preparation dried and examined.

Method of James.—James smears out an ordinary drop of blood so that it makes a circular smear about ¾ inch in diameter. This may be easily accomplished with a spatulate wooden toothpick. When dry, treat the blood smear with alcohol containing HCl (Alcohol, 50 cc.; HCl, 10 drops) until the haemoglobin is dissolved out. Then wash thoroughly in water for five or ten minutes. Allow to dry and then stain as ordinarily with the Wright or Giemsa stain.

Johnson's method.—Dry smear thoroughly without direct exposure to the sun. If the smear becomes discolored, as it may in hot weather when the time required is prolonged, it should be dehaemoglobinized with acid-alcohol. Prepare stain by adding 2 cc. of a 1% eosin solution to 220 cc. of distilled water and then adding 4 cc. of a 1% azur II solution, freshly made. The dried smears are flooded with the stain for twenty to thirty minutes. By this process the preparation is stained and the red cells are laked. The smears are then placed in distilled water until pink.

Ruge's method.—The best thick film method is that of Ruge. After the blood has dried well, gently move the slide about in a glass containing a 2% solution of formalin to which has been added 1% of glacial acetic acid. After laking is complete, as shown by disappearance of brown color, treat the slide in the same way in a glass of tap water to remove all traces of acid. Next wash very gently in distilled water and stain with dilute Giemsa (1 drop to 1 cc.) for twenty to thirty minutes. Wash in water and allow to dry without heat or blotting paper.

Some workers prefer to stain the dried thick smear for one hour in a jar containing dilute Giemsa stain (1 to 40) without previous fixation or dehaemoglobinization.

I make my thick films by taking up a very large loopful of the blood exuding from the puncture wound. This is deposited at one end of the slide and from it three or four daubs are made in succession toward the other end of the slide. These daubs are quickly smeared out before coagulation takes place in the first daub.

With all thick film methods it is extremely important to have thorough drying of the smear before dehaemoglobinizing or staining. This ordinarily requires one or two hours in the air, or twenty to thirty minutes in the incubator. It is particularly important in working with such smears, although holding for ordinary smears also, to protect them from flies, ants, etc., as such insects will eat up the smear in a few minutes if left exposed.

Fixation of Film.—In Wright's, Leishman's and other similar stains the methyl-alcohol solvent causes the fixation. In staining with Giemsa's stain, Ehrlich's triacid, haematoxylin and eosin, Smith's formol fuchsin, and with thionin, separate fixation is necessary. For Giemsa and thionin, either absolute alcohol (ten to fifteen minutes), or methyl alcohol (two to five minutes) answers well.

Formalin vapor, for five to ten seconds, is also used for fixation. For Ehrlich's triacid, haematoxylin and eosin and formol fuchsin, heat gives the best results. The best method is to place the films in an oven provided with a thermometer. Raise the temperature of the oven to 135°C. and then remove the burner. After the oven has cooled, take out the slides or slips which are then fixed.

Some prefer to place a crystal of urea on the slide, then hold it over the flame until the urea melts. This shows that a temperature between 130° and 135°C. has been reached.

One of the handiest methods is to drop a few drops of 95% alcohol on the slide or cover glass. Allow this to flow over the entire surface; then get rid of the excess of alcohol by touching the edge to a piece of filter paper for a second or two. Then light the remaining alcohol film from the flame and allow the burning alcohol to burn itself out. A chemical fixation which gives good fixation for haematoxylin and triacid stains (not equal to heat) is a modification of Zenker's fluid (Whitney). To Müller's fluid, which is potassium bichromate 2 grams, sodium sulphate 1 gram, and water 100 cc., add 5 grams of bichloride of mercury and 5 cc. of nitric acid (C.P.). Fixation is obtained in five seconds.

When using corrosive sublimate fixation one should after thorough washing in water treat the film with Gram's iodine solution for about two minutes and then wash with 70% alcohol until the yellow color of the film disappears. (See Staining of Protozoa.)

Staining of Dried Films

As separate staining with eosin and methylene blue rarely gives good preparations and as the modifications of the Romanowsky stain recommended are easy to make and employ, and give much more information, the separate method of staining is not recommended.

Rees' thionin solution.—The most satisfactory single stain is thionin. Take of thionin 1.5 grams, alcohol 10 cc., aqueous solution of carbolic acid (5%) 100 cc. Keep this as a stock solution. It should be at least two weeks old before using. For use, filter off 5 cc. and make up to 20 cc. with water.

1. Fix films (a) by heat, (b) by alcohol and ether, or (c) preferably by 1% formalin in 95% alcohol for one minute.

2. Stain for from ten to twenty minutes. Wash and mount. Malarial parasites are stained purplish; nuclei of leukocytes, blue; red cells, faint greenish-blue.

Ehrlich's triacid or triple stain.—There are required:

1. Sat. aq. sol. orange G. (Dissolve 3 grams in 50 cc. water.)
2. Sat. aq. sol. acid fuchsin. (Dissolve 10 grams in 50 cc. water.)
3. Sat. aq. sol. methyl green. (Dissolve 10 grams in 50 cc. water.)

These three solutions may be kept as stock solutions. They keep well in the dark. To make the stain, add 9 cc. of No. 2 (acid fuchsin) to 18 cc. of No. 1 (orange G.). After they are mixed thoroughly, add 20 cc. of No. 3 (methyl green). Then after these three ingredients are well mixed, add 5 cc. of glycerin. Mix, then add 15 cc. of alcohol; again mix, and finally add 30 cc. of distilled water. Keep the mixed stain about one week before using. The best fixatives are heat and Whitney's modified Zenker. To use, stain films from two to five minutes; then wash and mount. The triacid stain is a good tissue stain. The objections to the triacid stain are that it does not stain malarial parasites or mast cells and that failure to obtain good results is of frequent occurrence.

Romanowsky Stains.—In all methods of preparing Romanowsky stains, in which the polychroming of methylene blue is required, the specimen of dye used should be one known to be suitable for the purpose.

Wright's method.—The stain is made by adding 1 gram of methylene blue to 100 cc. of a 0.5% solution of sodium bicarbonate in water. This mixture is heated for one hour in an Arnold sterilizer. The flask containing the alkaline methylene blue solution should be of such size and shape that the depth of the fluid does not exceed 2½ inches. When cool, filter the methylene blue solution and add 500 cc. of a 1 to 1000 eosin solution (yellow eosin, water-soluble). Add the eosin solution slowly, stirring constantly until the blue color is lost and the mixture becomes purple with a yellow metallic luster on the surface, and there is formed a finely granular black precipitate. Collect this precipitate on a filter paper and when thoroughly dry (dry in the incubator at 38°C.) dissolve 0.3 gram in 100 cc. of pure methyl alcohol

(acetone-free). Wright lately has recommended using 0.1 to 60 cc. methyl alcohol. This constitutes the stock solution. For use filter off 20 cc. and add to the filtrate 5 cc. of methyl alcohol.

A modification by Balch is very satisfactory. In this method instead of polychroming the methylene blue with sodium bicarbonate and heat, the method of Borrel is used. Dissolve 1 gram of methylene blue in 100 cc. of distilled water. Next dissolve 0.5 gram of silver nitrate in 50 cc. of distilled water. To the silver solution add a 2 to 5% caustic soda solution until the silver oxide is completely precipitated. Wash the precipitated silver oxide several times with distilled water. This is best accomplished by pouring the wash-water on the heavy black precipitate in the flask, agitating, then decanting and again pouring on water. After removing all excess of alkali by these repeated washings, add the methylene blue solution to the precipitated silver oxide in the flask. Allow to stand about ten days, occasionally shaking until a purplish color develops. The process may be hastened in an incubator. When polychroming is complete, filter off and add to the filtrate the 1 to 1000 eosin solution and proceed exactly as with Wright's stain.

For collecting the precipitate, either for the Balch or the Wilson stain described below, we use a Buchner porcelain filter with stationary perforated disc. The stem of the filter is inserted into a doubly perforated rubber stopper which fits into a large flask. Glass tubing is passed into the other perforation and the filtration flask is connected up with a filter pump.

In Leishman's method the polychroming is accomplished by adding 1 gram of methylene blue to 100 cc. of a 0.5% solution of sodium carbonate. This is kept at 65°C. for twelve hours and allowed to stand at room temperature for ten days before the eosin solution is added. The succeeding steps are as for Wright's stain.

Wilson's stain.—Although the reagents from which this stain is prepared are not used in the same quantities as in making up the Balch stain, the two stains employ the same polychroming principle and are used in the same way. The Wilson stain appears to have more of the rich-staining characteristics of the Giemsa than does the Balch.

The silver oxide is prepared according to the method given under Balch's modification except that 2 grams of silver nitrate are used. Two solutions are then prepared as follows:

Solution I. To the moist silver oxide add 2 grams of methylene blue dissolved in 200 cc. of a 0.5% solution of sodium bicarbonate in distilled water.

Gently boil this silver oxide methylene blue mixture in a rather deep porcelain dish for thirty minutes, stirring occasionally. Pour off and preserve about one-third of the contents of the dish into a 200-cc. graduated cylinder. Add to the contents of the dish an amount of boiling distilled water, equal to that poured into the cylinder, and boil again for thirty minutes. Again pour off and preserve one-third the contents of the porcelain dish. Then boil the remaining contents of the dish for another thirty minutes, not adding additional water. Now add the contents of the dish to the portions previously preserved and set aside and make up the total volume with water to 200 cc. Filter through a coarse filter into a 500-cc. beaker and immediately add to it:

Solution II. For this solution dissolve 1 gram of yellow water-soluble eosin in 200 cc. distilled water.

Allow the mixture of the two solutions to stand about thirty minutes and then filter, collecting the precipitate on a hard filter paper. The precipitate may be dried in an incubator or hot air oven at 60°C. The yield of dry precipitate is about 1.7 grams.

For the staining fluid dissolve 0.2 gram in 50 cc. pure methyl alcohol.

Terry's simplified method for preparing a modified Romanowsky stain.—A solution of 1 per cent. pure methylene blue and of 0.5 per cent. anhydrous sodium carbonate in distilled water is brought to a boiling point in a porcelain dish and kept gently boiling for two and a half minutes. It is then cooled and 500 cc. of a 0.1 per cent. solution of yellowish water-soluble eosin is slowly added. A precipitate forms and is separated off by filtration and dried.

A half-saturated solution of the dried precipitate prepared as above in pure methyl alcohol (free from acidity and acetone) is used for staining blood films. The pure stain is allowed to act on fresh unfixed films for one and a half minutes. It is then diluted with 2 volumes of distilled water and allowed to act for three minutes longer. The films are finally washed with distilled water.

The purity of the methyl alcohol is of great importance, as the polychrome stain loses all its best qualities if dissolved in an alcohol containing the least trace of impurity. The distilled water should be neutral.

Giemsa's modification of the Romanowsky method.—This is one of the most perfect of the modifications. The objection is that greater time in staining films is required than with the Wright or Leishman method and the stain is very expensive.

Take of Azur II eosin 0.3 gram. Azur II 0.08 gram.

Dissolve this amount of dry powder in 25 cc. of pure anhydrous glycerin at 60°C. Then add 25 cc. of methyl alcohol at the same temperature. Allow the glycerin methyl-alcohol solution to stand over night and then filter. This is the stock stain. To use: Dilute 1 cc. with 10 to 15 cc. of distilled water. If 1 to 1000 potassium carbonate solution is used instead of water it stains more deeply.

The alkaline diluent is used to obtain the coarse stippling in malignant tertian (Maurer's clefts). Having fixed the smear with methyl alcohol for one to five minutes, pour on the diluted stain, and after fifteen to thirty minutes wash off and continue washing with distilled water until the film has a slight pink tinge. For *Treponema pallidum* stain from two to twelve hours.

French's modification of Giemsa's stain.—A substitute for Giemsa of the following composition is offered. This advantage is claimed: it has certain definite components and can be consistently prepared.

Eosin	1.250 Gm.
Methylene blue	1.250 Gm.
Methylene azure A (MacNeal)	0.750 Gm.
Methylene violet (Bernthsen)	0.250 Gm.
Methyl alcohol, absolute	375.0 cc.
Glycerin (anhydrous)	125.0 cc.

The proportion of the components is the same as that used by MacNeal in the tetrachrome stain though the concentration is much greater to allow of the customary Giemsa technic. These proportions give the correct blue elements for a complete differential staining of the basic components.

While the stain can be prepared by the above formula, some difficulty may be experienced unless a special quality of glycerin is used—the usual samples of glycerin are not sufficiently dry. It is found that this quality in the glycerin is an important consideration.

Another Giemsa substitute.—Methylene blue, 0.75 Gm. Dissolve in (95% alcohol, 50 cc., glycerin 50 cc. and 10% Na_2CO_3 3 cc.). Boil 15 minutes. Add 1% eosin 35 cc. Boil 15 minutes and add alcohol to 100 cc. Stopper. Let stand a week.

Tetrachrome stain.—Under this designation, one may now purchase the four active staining agents present in the Giemsa stain, methylene blue, eosin, methylene azur and methylene violet, combined and ready for use. The stain is prepared by dissolving 0.3 gram in 100 cc. pure methyl alcohol. It stains cells and protozoa exceptionally well, and appears to be much less sensitive to variations in reaction of the diluting water than is the Balch stain.

The diluting water.—In all Romanowsky methods distilled water, CO_2-free, should be used (see p. 687). If not obtainable, the best substitute is rain water collected in the open and not from a roof.

If the yellow color in water in a test tube to which has been added a small pinch of haematoxylin does not change to blue in from one to five minutes it is too acid and should be treated with a 1% sod. carb. sol. until it does show blue. Alkaline waters are less easy to correct. There is often a high initial acidity of distilled water which can be lowered by boiling so that it will act satisfactorily as a diluent. When a Wright stain becomes unsatisfactory by reason of too intense basic staining one may rectify the defect by adding drop by drop N/50 HCl and trying out the staining characteristics with a number of smears. In doing this one should always use a standard distilled water (freshly boiled).

Another method to adjust the distilled water to the right reaction is to measure the amount of water required (say 5 cc. for staining two or three films) into a clean test-tube. With a glass rod add a very small drop of neutral red solution (1 per cent. in distilled water), and stir. The water will turn pink (acid). Now add, with the glass rod, a trace (not a drop) of 1 per cent. aqueous sodium carbonate solution, and stir again. If the solution turns a pinkish yellow it is now neutral; if it remains pink it is still acid, and a further trace of carbonate must be added, and the process repeated till the right color is obtained; if it turns yellow, it has become alkaline, and too much carbonate has been added. (In this last case it is best to discard it, and begin again.) The color to be aimed at is one between red and yellow, and with a little practice it can usually be hit off in a few seconds. When the water shows the right reaction, as thus determined by its color, it is ready for use.

Technique of staining (except for Giemsa).—(1) Make films and air-dry.

2. Cover dry film preparation with the methyl-alcohol stain for one minute (to fix).

3. Add water to the stain on the cover glass or slide, drop by drop, until a yellow metallic scum begins to form. It is advisable to add the drops of water rapidly in order to eliminate precipitates on the stained film. Practically, we may add 1 drop of water for every drop of stain used.

4. Wash thoroughly in water until the film has a pinkish tint.

5. Dry with filter paper and mount. The stained preparation is less apt to show foreign material and damage if one allows the film to dry without blotting. In a moist atmosphere one may dry the film high over the flame but any near contact with the heat of the flame is injurious.

Appearance of stained cells.—Red cells are stained orange to pink; nuclei, shades of violet; eosinophile granules, red; neutrophile granules, yellow to lilac; mast cell granules, deep violet; blood platelets, purplish; malarial parasites, blue; chromatin, metallic red to rose pink.

By using a 1% solution of sodium carbonate instead of distilled water for diluting the Wright stain various spirochaetes, even treponemata, are quite intensely stained.

The bottle in which the methyl alcohol solution is kept must be tightly stoppered with a cork stopper and kept in the dark. Any evaporation of the alcohol interferes with proper fixation of the blood film and the light affects the delicate stain.

Haematoxylin Staining.—While the Romanowsky methods are more satisfactory for differential counts and for the demonstration of malarial parasites, and especially for differentiating species, yet by reason of the liability of methylene blue to deterioration in the tropics the haematoxylin methods may be preferable. Many workers in blood work and cytodiagnosis prefer the haematoxylin.

1. Fix the film either by heat, with methyl alcohol for two minutes or with Whitney's fixative. Heat is to be preferred.

2. Stain with Meyer's haemalum or Delafield's haematoxylin for from five to fifteen minutes according to the stain. Frequently three minutes will be found sufficient. To make the haemalum, dissolve 0.5 gram of haematin in 25 cc. of 95% alcohol. Next dissolve 20 grams of ammonia alum in 500 cc. of distilled water. Mix the two solutions and allow to ripen for a few days.

3. Wash for two to five minutes in tap water to develop the haematoxylin color.

4. Stain either with a 1 to 1000 aqueous solution of eosin or with a 0.5% eosin solution in 70% alcohol. The eosin staining requires only fifteen to thirty seconds.

5. Wash and examine.

Coagulation Rate, Viscosity, Iodophilia, Red Cell Fragility and the Peroxidase Reaction

Iodophilia.—This reaction is supposed to be due to the presence of glycogen, especially in the polymorphonuclears, in suppurative conditions. At present this test is rather discredited.

It has been stated that a differentiation between the joint involvement in gonorrhoeal infection and in articular rheumatism may be made from iodophilia being present in the gonococcus infection.

Make blood smears on cover glasses as usual, and after they dry, but without fixation, mount them in a drop of the following solution:

Iodine	1 part.
Potassium iodide	3 parts.
Gum arabic	50 parts.
Water	100 parts.

Small brown masses in the polymorphonuclears indicate a positive iodophilia.

Volume Index.—Capps introduced the term *volume index* to designate the relation existing between the volume of the red cells determined by the haematocrit and their total count. The volume index as a rule corresponds closely to the color index, and variations have the same significance.

For clinical estimation of the blood volume the haematocrit tube is used. The method of using the Van Allen haematocrit tube is as follows:

A mouthpiece with connecting tube is attached to the tube above the bulb, and a drop of fresh blood from a skin puncture, either finger or ear, is drawn into the capillary exactly to the top of the scale. A small quantity of the anticoagulant fluid, which consists of 1.3% sodium oxalate solution, is drawn up with the specimen until the bulb is about one-third full. The lower end is stoppered with the finger until the rubber sealing band, or preferably the spring sealing clip, is attached. Centrifugalization is now performed for about twenty minutes at 3000 r.p.m. Reading of the relative corpuscle volume is then made from the scale directly in percentage.

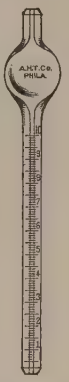


FIG. 82.—Van Allen haematocrit tube.

Make a red corpuscle count and express the result in percentage, taking 5,000,000 as normal. Then divide the percent of red cells by volume by the per cent of red cells obtained by counting. The result is the volume index.

Viscosity of the Blood.—This is estimated by observing the relative height to which blood rises in capillary tubes as compared with water, and normally varies from three to five. The higher the haemoglobin content the greater the viscosity. Viscosity is high in arteriosclerosis and diabetic coma, low in the anaemias of nephritis.

Coagulation Rate of Blood.—This determination is of value in connection with operations on jaundiced patients and in the detection of a haemophilic tendency.

A delay in the coagulation rate is observed in haemophilia, certain cases of pernicious anaemia, splenic anaemia and the leukaemias—all these being diseases in which spontaneous haemorrhage occurs. An accelerated coagulation rate in typhoid fever indicates the occurrence of thrombosis.

There are many points to consider in estimating coagulation time. Much depends on the character and location of the vessel severed; for example, a bleeding minute vessel in a Peyer's patch may fail to form a clot, while a far larger superficial artery may cease to bleed before serious loss of blood occurs. Then, too, the rate of clotting varies with the time of day, coagulation being slowest in the forenoon and quickest about 5 o'clock in the afternoon. When the blood for the test is obtained from a finger or ear, it is standard practice to use the second or third drop, as each succeeding drop clots more rapidly than those preceding.

It has now been well established that the proper source of the blood for testing coagulation rate is a vein.

Sabrazes' method.—A simple method of determining the rate is to take a piece of capillary glass tubing and hold it downward from the puncture to let it fill for 3 or 4 inches. Then at intervals of thirty seconds scratch with a file the capillary tubing at short distances and break off between the fingers. When coagulation has taken place a long worm-like coagulum is obtained. Normally coagulation occurs in about three to four minutes, when the temperature is that of the hand in which the tubes are conveniently held. Rudolf recommends placing the tubes in metal tube containers in a Thermos bottle at 20°C. He gives the normal coagulation rate for this temperature as eight minutes, while at a temperature below this the period is lengthened. Age and sex do not influence the rate. Sabrazes found no appreciable variation in tubes from 0.8 to 1.2 mm. diameter.

One can attach a piece of glass tubing, the end of which has been drawn out into a capillary tube, to a hypodermic needle by a short rubber connection. See Fig. 10. Insert the needle into the vein and fill the capillary tube. File off as above. From the vein, at room temperature the coagulation rate is from six to eight minutes. From the ear or finger three to four minutes.

In *Bürker's test* you mix a drop of blood and a drop of distilled water on a slide and with a capillary tube sealed off at the end stir the mixture every half minute. So soon as fibrin threads appear you have coagulation.

Method of Lee and White.—Take a sterile 1-cc. all-glass syringe and a small test tube 8 mm. in diameter. Rinse both with sterile saline. Introduce the needle into a vein and withdraw exactly 1 cc. of blood which is immediately forced out into the test tube. Tip the tube every 15 to 20 seconds. When the blood no longer flows, but solidifies so that the surface contour remains fixed when the tube is tilted or even inverted, we read the time as that of coagulation. This averages about 6 to 7 minutes.

Character of the clot.—Normally the clot begins to retract a few hours after it forms, and separates from the side of the containing vessel, expressing the serum. The retraction is completed in from 18 to 24 hours. To study this phenomenon a few cc. of blood are placed in a test tube, kept in an incubator at 37°C., and observed at intervals. Retractility appears to be due to the presence of blood platelets and is entirely independent of coagulation time. In thrombocytopenia the clot is non-retractile, while in haemophilia the clot retracts normally, although the coagulation may be greatly delayed.

Bleeding time.—For this determination we use Duke's method. Make a deep prick in the lobe of the ear, and, at intervals of 30 seconds, take up the drops as they exude with a piece of filter paper, care being exercised to avoid touching the

skin. Note the time elapsed when clotting occurs. Normally the continuance of bleeding is not more than two or three minutes. The bleeding time is greatly increased in thrombocytopenia and in chloroform and phosphorus poisoning. In haemophilia the bleeding time is usually not prolonged.

Fragility of Red Cells.—The fragility of the red cells is increased to quite a degree in haemolytic jaundice. Pernicious anaemia shows increased resistance and even in purpura the resistance to haemolysis is normal. In chronic obstructive jaundice the resistance is increased somewhat.

With normal blood, haemolysis begins in .45 to .38% NaCl and is complete in solutions of 0.36 to 0.32%. It is well in testing fragility to use the blood of a normal individual as a control.

We have found the technique of Griffin and Sanford to be very satisfactory. Mark twelve $3 \times \frac{3}{8}$ inch test tubes with numbers from 25 to 14. Into each tube drop as many drops of 0.5% NaCl as the number on the tube. With the same pipette make up the volume in each tube to 25 drops by adding drops of distilled water. Tube 14 would require 11 drops. To obtain the NaCl percentage of each tube multiply the number on it by 0.02. To each tube add one drop of blood just drawn from a vein with a sterilized all-glass syringe, which should be dry. Add one drop of whole blood to each tube and mix thoroughly. A similar procedure should be employed for the blood of the normal control. Allow the tubes to stand for one or two hours at room temperature and then read the results.

The Peroxidase Reaction.—In differentiating leukocytes of marrow origin from those of lymphatic origin the absence of very deep-blue-staining granules in the latter is of value. In myeloblasts we note these darkly stained granules which are absent in lymphoblasts.

We have used a modification of Goodpasture's stain with satisfaction. The stain is prepared as follows:

Sodium nitroprusside	0.05 Gm.
Benzidine (C.P.) (Harmer)	0.05 Gm.
Basic fuchsin	0.10 Gm.
Alcohol (95%)	100.00 cc.

Dissolve the nitroprusside in as little water as possible (1 to 2 cc.) and add to the alcohol. Then dissolve the other reagents. Make fresh blood smears on a slide and allow to dry thoroughly. Cover the slide with the stain and allow one minute for fixation. Then add an equal quantity of 1:200 dilution of hydrogen peroxide in distilled water. This gives the same concentration of H_2O_2 as in the original Goodpasture stain. Let the diluted stain act for three minutes. Wash and dry.

The granules of polymorphonuclears and eosinophiles are stained an intense blue. Basophile granules do not appear to stain. Myelocytes and myeloblasts show deep-blue granules. Lymphocytic cells stain a light red and red cells a buff color. The large mononuclears and transitionals show a few blue-black granules.

Specific Gravity of the Blood

Hammerschlag has a method for the determination of the Hb. percentage based upon the specific gravity of the blood.

In this method a mixture of benzol and chloroform is made of a specific gravity of about 1.050. A drop of blood is then taken up with a pipette and introduced below the surface of the mixture, carefully avoiding production of bubbles. Variation in temperature introduces a very appreciable error. If it sinks, add more chloroform from a dropping bottle; if it tends to rise, more benzol. The mixture in which the drop of blood tends to remain stationary, near the top of the mixed benzol and chloroform, has the same specific gravity as that of the blood. This is determined by an accurately graduated hydrometer. The normal average specific gravity for men is 1.059, for women 1.056. A table, giving the Hb. percentage corresponding to the specific gravity, accompanies the outfit.

The specific gravity is reduced in all anaemias, especially chlorosis, and in nephritis with oedema as well as in most cachectic states. In these latter the Hb. percentage may be normal.

Specific gravity in cholera.—To determine the necessity for intravenous infusion in cholera Rogers has recently recommended the employment of small bottles containing aqueous solutions of glycerin with specific gravities varying from 1048 to 1070, increasing the specific gravity in each successive bottle by 2°.

Drops of blood from the cholera patient are deposited at the center of the surface of the fluid in the bottles from a capillary pipette. If the specific gravity of the blood is 1062 at least a liter of saline or sodium bicarbonate solution is needed. If 1066 at least 2 liters. Formerly he estimated the indications by blood pressure, considering a pressure of 80 in Europeans or of 70 in natives as indicating intravenous injections.

Sedimentation Test.—The sedimentation test consists in observing the rapidity with which the red blood cells settle out from the plasma. *Fahraeus* was one of the first to make observations upon the rate of sedimentation of red blood cells in citrated blood. He used the method of vein puncture, running the blood directly from the needle into a small tube containing 2 cc. of 2% sodium citrate solution. The tube is 17 cm. long, with an internal diameter of 9 mm., and there is a mark indicating a content of 10 cc. about 2 cm. from the top of the tube, to which the blood is run. The contents are mixed by inversion, and sedimentation rate is estimated by the depth of the clear fluid which appears on the top, at the end of one hour.

In *Linzenmeier's method,* the tubes are 3 to 4 mm. in diameter and 6.5 cm. high, with a mark indicating the level of 1 cc. of fluid. This mark is indicated by the figure 1, and below this marks are made at 6, 12, 18, and 24 mm. respectively. A 3.8% sodium citrate is used, 0.2 cc. being drawn into the syringe and then the syringe filled up to 1 cc. with blood obtained by venepuncture. The citrated blood is mixed and placed in the tube and the time noted for the upper level to fall to the 6, 12 and 18 mm. mark. In a normal individual it takes about five hours for the red cells to sediment to the 18 mm. mark.

Westergren's method is more elaborate and his observations were chiefly on the sedimentation rate in tuberculosis.

Cutler's method appears the simplest and best of the various methods and we have found it very satisfactory. The only essential apparatus required is a special sedimentation tube of at least 5 cc. capacity, graduated into tenths of a cubic centimeter and marked in millimeters. Cutler's procedure is as follows:

Aspirate into syringe 0.5 cc. of 3 per cent. sodium citrate solution; puncture vein and draw blood to 5 cc. mark. Draw back the barrel of the syringe about 1 cm. and gently tilt the syringe backward and forward several times to insure uniform mixing of blood and citrate solution. Remove needle from syringe, as it may contain clotted blood, and pour contents into the sedimentation tube. When it is necessary to obtain specimens from several patients, let this tube stand in the rack without paying any attention to it. Wash syringe in water, wipe barrel with a clean cloth and then pass syringe through alcohol and ether and dry in air; this insures a clean and dry syringe which is again ready for use. After all the samples of blood have been taken they are brought the laboratory. It is best to number each tube to avoid possible error, and carry the tubes in a rack. Before any readings are made each tube is stoppered with a paraffin-coated cork and gently turned upside down two or three times; this insures a uniform distribution of the red blood cells, for by the time the tubes are brought to the laboratory, in many cases marked sedimentation has already taken place.

The tubes can be allowed to stand as long as ten hours before making any readings. After ten hours the sedimentation phenomenon begins to disappear. The position of the recording column of blood is read every five minutes for one hour, and the observations are recorded on special sedimentation charts on which the horizontal lines represent the divisions on the sedimentation tube and the vertical lines, the intervals of time. In this way a graph is traced which shows the position of the sedimenting column of red blood cells at any period during the first hour.

The normal sedimentation index for men varies from two to eight mm., with an average of three to four; for healthy women from two to ten mm., with an average of five to six; during menstruation probably as high as twelve mm.

It must be understood that the sedimentation test is not a special test for any disease. It does appear, however, that the test may have some value in such a disease as tuberculosis. Westergren states that the normal sedimentation rate is never obtained in active tuberculosis.

Cutler's conclusions in regard to the value of this test in tuberculosis are summed up as follows:

As an aid in estimating activity, the sedimentation test is more reliable than temperature curve, pulse rate or gain in weight, the three major guides in the treatment of tuberculosis.

By repeating the test at regular intervals, the true course of the disease can be graphically represented, for as the individual improves the graph should approach more and more the horizontal line, but should he become worse, more and more the vertical curve.

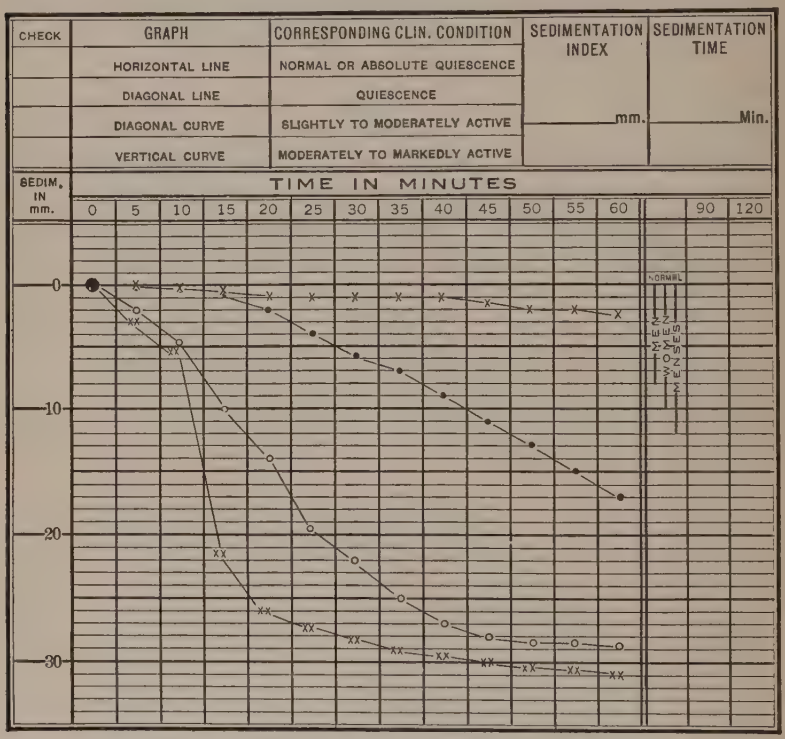


FIG. 83.—Cutler's graphic chart for recording Blood Sedimentation. (*American Journal of the Medical Sciences, June* 1926.)

———×———×———Horizontal line (clinically healthy individual).
———●———●———Diagonal line (clinically quiescent tuberculosis).
———○———○———Diagonal curve (clinically slightly active tuberculosis).
———××———××———Vertical curve (clinically markedly active tuberculosis).

Enough clinical evidence has been published by various individuals using diverse technique to warrant the adoption of this test as a routine procedure in institutions responsible for the care of tuberculous patients.

The sedimentation test is a nonspecific reaction, occurring in many infections and destructive diseases and should be studied extensively so that its limitations and probable value could be fully established.

The graphic method is the most informative method that has yet been proposed for the study of the blood sedimentation test and should be the method of choice in all future investigations.

Tests for Agglutination and Haemolysis of the Red Cells (Transfusion)

Blood transfusion has been so simplified by the various techniques of indirect transfusion, using citrated solutions for preventing the coagulation of the blood, that simple laboratory methods for determining the suitability of the blood of donors have become of immense importance.

In war surgery, in the treatment of cases in which there has been great loss of blood, transfusion is an important therapeutic measure. It is indicated not only in traumatic haemorrhages but, as well, in those anaemias resulting from prolonged loss of blood, in carcinoma, alimentary tract ulcerations, etc. This measure is also advocated in pernicious anaemia. The best results seem to follow its use in the treatment of mēlena neonatorum. Although injection of sodium bicarbonate is the measure usually recommended in severe cases of blackwater fever yet blood transfusion should be kept in mind.

Classification of Human Blood into Four Groups.—In 1900 Landsteiner found that, when serum of certain individuals was mixed with cells of other individuals, agglutination occurred. From his observations he was able to divide human blood into three groups with respect to iso-haemagglutination. In 1902 Decastello and Sturli described a fourth group. In 1907 Jansky classified the four groups as follows:

Group I, the serum of which agglutinates the corpuscles of Groups II, III and IV, while the cells are not agglutinated by any serum.

Group II, the serum of which agglutinates the corpuscles of Groups III and IV, but not those of Groups I and II, while the corpuscles are agglutinated by the serum of Groups I and III, but not by those of Groups II and IV.

Group III, the serum of which agglutinates the cells of Groups II and IV, but not those of Groups I and III, while the corpuscles are agglutinated by the serum of Groups I and II, but not by those of Groups III and IV.

Group IV, the serum of which does not agglutinate any corpuscles, while the corpuscles are agglutinated by the serum of all other groups.

JANSKY'S CLASSIFICATION

CELLS OF GROUPS	SERUM OF GROUPS I	II	III	IV
I	−	−	−	−
II	+	−	+	−
III	+	+	−	−
IV	+	+	+	−

SERUM OF GROUPS	CELLS OF GROUPS I	II	III	IV
I	−	+	+	+
II	−	−	+	+
III	−	+	−	+
IV	−	−	−	−

Independently of Jansky, Moss made the following classification in 1910:

Group I, the serum of which does not agglutinate any corpuscles, while the corpuscles are agglutinated by the serum of Groups II, III and IV.

Group II, the serum of which agglutinates the corpuscles of Groups I and III, while the corpuscles are agglutinated by the serum of Groups III and IV.

Group III, the serum of which agglutinates the corpuscles of Groups I and II, while the corpuscles are agglutinated by the serum of Groups II and IV.

Group IV, the serum of which agglutinates the corpuscles of Groups I, II and III, while the corpuscles are not agglutinated by any serum.

MOSS' CLASSIFICATION

CELLS OF GROUPS	SERUM OF GROUPS I	II	III	IV
I	−	+	+	+
II	−	−	+	+
III	−	+	−	+
IV	−	−	−	−

SERUM OF GROUPS	CELLS OF GROUPS I	II	III	IV
I	−	−	−	−
II	+	−	+	−
III	+	+	−	−
IV	+	+	+	−

The two classifications given are in common use. Although that of Moss is more generally followed in France, England and the United States, the obvious desirability of having one classification universally employed, in order to avoid confusion and the possibility of serious accidents, has led to the recommendation that, on the basis of priority, the groupings of Jansky be adopted. The Jansky and Moss classifications differ in that Groups I and IV have been interchanged. Groups II and III are the same.

It has been generally accepted that the four groups considered include all adult persons; i.e., that the classification is complete. Recent research, however, indicates the existence of other groups. (See below.)

Moss' original report, based on 1600 tests, gave the following incidence—expressed in Jansky's classification: Group I, 43%; Group II, 40%; Group III, 7% and Group IV, 10%.

Culpepper and Ableson have typed 5000 bloods using Moss' groupings. Transferred to the Jansky classification their results are as follows: Group I, 44.48%; Group II, 36.06%; Group III, 14.28% and Group IV, 5.18%.

Karsner has compiled the findings of five different investigators and on the basis of more than 5000 tests the average incidence (Jansky classification) is as follows: Group I, 42.84%; Group II, 41.38%; Group III, 10.36% and Group IV, 5.42%.

Lewis and Henderson brought out a very interesting fact regarding distribution of the blood groups in various races. Amongst the European races Group II is 2.5 to 4.5 times as common as Group III, while among the Asia-African races, including Malagasies, Negroes, Anameses and Hindus, Group II and Group III become more equal in number, Group III at times even exceeding Group II. Turks, Arabs, Russians and Jews form an intermediate group in which the incidence of Groups II and III lies somewhere between those of the other race groups.

Immune substances responsible for the reaction.—In order to explain these phenomena of iso-agglutination there is assumed the existance of two iso-agglutinins, a and b, in sera: one, a, being in Group III, the other, b, in Group II, both in Group I, and neither in Group IV.

According to the same theory two receptors for these agglutinins, iso-agglutinogens, A and B, are supposed to be distributed among the corpuscles of the groups in such fashion that an agglutinin and its corresponding agglutinogen are never present simultaneously in the same blood. In Jansky's grouping we find the following distribution of these substances.

	Agglutinin (Serum)	Agglutinogen (Cells)
Group I	a b	– –
Group II	– b	A –
Group III	a –	– B
Group IV	– –	A B

From this table it is easily seen that the serum of Group I, containing both a and b agglutinins will agglutinate cells from all other groups as the cells from each one of these groups contain one or both of the agglutinogens (A and B). The cells of Group I, not containing any agglutinogens will not be agglutinated by any of the sera. Group IV presents the opposite.

In addition to the iso-agglutinins and iso-agglutinogens there are also present normally in the blood iso-haemolysins and iso-haemolysinogens. Until recently it has been supposed that these immune substances act interdependently and that haemolysis cannot occur without agglutination. Kolmer has shown, however, that the agglutinins and the haemolysins are separable by heat and that haemolysis is not necessarily preceded by agglutination. For all practical purposes, however, the agglutination tests as described below suffice for the determination of suitability of donors for transfusion. Only about one-fifth of agglutinating sera prove also haemolytic.

Holt and Reynolds have shown that the haemagglutinating property is contained in the pseudoglobulin fraction of the serum and that the euglobulin and albumin apparently play no part in the reaction.

It appears that diet has an influence on certain of the reactions by means of which we determine the "group" to which an individual belongs, and it seems quite possible that other factors may act similarly. For example, Harper and Byron have observed that the lack of sufficient green vegetables in the diet reduces the agglutinating power of Group III serum, and, further, prevents the proper agglutination of Group III cells by Group II serum, while reactions involving the sera and cells of the other groups are not affected.

Guthrie and Huck have apparently shown that the blood grouping as presented above is not complete. They have found two types of blood that do not conform with this classification and by various absorption experiments have shown the probability of the existence of a third agglutinogen and a third agglutinin which they term C and c respectively. According to this classification the old Group II must be subdivided into two subgroups, the sera of which contain agglutinin a but the red cells of which contain the agglutinogens A and AC respectively. Group III must also be subdivided into two subgroups the red cells of which contain the agglutinogen A but the sera of which contain the agglutinins ac and c, respectively.

Autoagglutination and autohaemolysis.—Rarely in pernicious anaemia it will be found that a patient's serum may agglutinate his own red cells. This autoagglutination is regarded as an important diagnostic test in acquired haemolytic jaundice. In paroxysmal haemoglobinuria, a chronic disease often due to syphilitic infection, there develops in the blood serum a specific haemolysin capable of producing autohaemolysis following exposure to cold. Haemolysis may be brought about outside the body when the serum is chilled, and subsequently warmed and mixed with either the patient's cells or those of a normal individual.

When the haemoglobinuric blood is stored for 30 minutes or longer at 0°C., the complement becomes so altered, according to Yorke and Macfie, that at the subsequent heating haemolysis will not take place without the addition of fresh complement. Hence blood to be used for the test should be used immediately after collection. Further, when the haemoglobinuric serum is to be used against cells from a normal individual, it should be separated immediately, since its own red cells will, on prolonged contact at 0°C., absorb the haemolytic immune body, thus leaving the serum inactive. This absorption of amboceptor is shown, not only by the failure of haemoglobinuric serum under these conditions to haemolyze normal cells, but also by the fact that the haemoglobinuric cells, having become "sensitized," are haemolyzed by normal serum (complement).

The property of developing autohaemolysins upon chilling and subsequent heating is not possessed in such degree by the serum of any

other disease—certainly not by that of blackwater fever, the disease with which paroxysmal haemoglobinuria is most readily confused. The test demonstrating this autolysis in vitro thus becomes practical and important from a diagnostic point of view. The method of Yorke and Macfie is performed as follows:

Serum 6 volumes	Cells 4 volumes	Incubation	Result
Patient's	Patient's	0°C. for 5 to 7.5 minutes then 37°C. for one hour.	Haemolysis
Patient's	Normal		Haemolysis
Normal	Patient's		No haemolysis
Normal	Normal		No haemolysis

When haemoglobinaemia exists, the liver converts the haemoglobin into bile pigments; but if so many as one-sixth of the red cells are destroyed haemoglobinuria results.

Testing Blood for Compatibility.—In the selection of a donor for blood transfusion it is always necessary to try his red cells against the serum of the recipient as well as the patient's red cells against the serum of the donor, in order to prove the absence of agglutinating or haemolyzing bodies. As seen under the discussion of blood groupings, the cells of Group I (Jansky) are not agglutinated by any sera; hence individuals of this group may serve as "universal donors" in spite of the fact that the cells of the recipient's blood are agglutinated by the donor' serum, the danger of blood transfusion lying in the agglutination of the donor's cells by the recipient's serum. Conversely one may speak of individuals of Group IV (Jansky) as "universal recipients." When possible, however, the group of the donor's blood should always be the same as that of the recipient. It must be borne in mind that the "group" of an individual may change after an infusion, and hence the test for compatibility should be carried out preceding every transfusion.

It is important to do a Wassermann or Kahn test on donors.

Before transfusing carry out one of the following tests:

1. From a vein aspirate 3 or 4 cc. of blood and add 0.5 cc. to a centrifuge tube containing 4.5 cc. of 1% sodium citrate in normal salt solution. The remainder is placed in another tube and the blood serum separated. Throw down the cells in the citrated blood by centrifugation, pipette off the supernatant fluid and wash the cells by adding normal saline. After centrifuging, again pipette off saline and make a 10% suspension of cells by making up to original volume with normal saline.

Carry out these procedures for both donor and recipient.

Tests: (1) In a small test tube deposit 1 drop of the donor's 10% red-cell emulsion and then add 4 drops of the recipient's serum.

(2) Treat similarly 1 drop of the recipient's red-cell emulsion with 4 drops of the donor's serum.

(3) Treat 1 drop of donor's red-cell emulsion with 4 drops of his serum.

(4) Treat 1 drop of recipient's red-cell emulsion with 4 drops of his serum.

Finally add 1 cc. of salt solution to each of the 4 tubes, shake gently and place in incubator for two hours.

(5) Treat 1 drop of donor's red-cell emulsion with 4 drops of salt solution.

(6) Treat 1 drop of recipient's red-cell emulsion with 4 drops of salt solution.

Tubes 5 and 6 are controls of saline.

Tubes (3) and (4) should fail to show either agglutination or haemolysis. If agglutination or haemolysis appears in tubes 1 or 2, the donor is not satisfactory; but if agglutination appears in tube 2 only, he may be used in an emergency.

Some prefer to keep the tubes over night in ice box after the preliminary examination following incubation.

2. *Lee's technique.*—For the regular carrying out of this method one should keep on hand the sera of individuals belonging to Groups II and III. To carry out the tests prepare a suspension of the donor's red cells by dropping 2 or 3 drops of his blood into 1 cc. of citrated salt solution. Deposit a platinum loopful of standard Group II serum on a slide and emulsify in it a loopful of the donor's red-cell suspension. A concave slide with two concavities is convenient, the serum-cell emulsion being made on the cover glasses which are to be inverted over the vaseline-ringed concavities. The agglutination can be observed with a high power magnifying glass or the $\frac{2}{3}$-inch objective. Agglutination, when it occurs, is usually complete in five to fifteen minutes. Repeat test with Group III serum.

If both test sera agglutinate the donor's cells he belongs to Group IV (Jansky). If neither agglutinates, to Group I (Jansky). Agglutination by Group II serum but not by III puts the donor in Group III. Agglutination by Group III serum but not by Group II shows a Group II donor. If an individual belonging either to Group II or Group III, is available it is possible to determine the grouping of a large number of other individuals by trying his serum and cells against the cells and sera of the unknown.

For example: An individual of Group II is available. His cells are tried against the serum of an unknown. We find that agglutination occurs. By referring to the Jansky classification it is seen that the unknown must belong to either Group I or Group III. The cells of the unknown individual are now tried against the serum of the known Group II individual. Agglutination occurs. By again referring to the table we find that the unknown belongs to Group III.

Ottenberg, recommends the open slide method of Vincent. One drop of serum is placed on a plain slide, and into it is allowed to fall 1 drop of the cell emulsion. (This is better than platinum loopfuls of serum and cells, because with the latter the amount is rather too small.) The slide is tilted and rotated gently, so that the cells are uniformly distributed; this is repeated every two minutes. Agglutination is easily seen with the naked eye in from one to ten minutes at room temperature. The microscope is not needed and should not be used. Genuine

agglutination is always visible to the naked eye. The observations should never be extended for longer than fifteen minutes.

3. *Lee's emergency method.*—When standard sera II and III are not on hand one may use the following method:

"A small amount of blood is collected from a patient (1 cc. from the ear or finger is sufficient), and allowed to clot. The serum is then obtained. One drop of this serum is placed on a slide and mixed with a drop of suspension of blood of the donor taken into 1.5% citrate solution. (A few drops of blood are taken into approximately 10 times the amount of 1.5% citrate solution and shaken. It is very important that the blood be dropped directly into the citrate, and should not be partially coagulated.) In the event of a positive test, marked agglutination will be evident in a few moments and is easily seen macroscopically. In the event of a negative test it is a wise precaution to raise the cover glass, and after making sure that the serum and cells are well mixed, to examine the preparation again. The only possible source of confusion is the appearance of rouleaux of the red corpuscles, indicating a too thick emulsion. If the test is negative, transfusion may be regarded as entirely safe."

Preparation of stock serum.—This is done by bleeding Group II and Group III individuals whose groups are determined with the original serum. The serum is separated and to each nine (9) cc. of the clear serum is added one (1) cc. of fifteen per cent. sodium citrate solution in physiological salt solution, making a dilution of 1.5% citrate solution in the serum. To each nine (9) cc. of this citrated serum is then added one (1) cc. of a five (5) per cent. solution of phenol in neutral glycerin. This gives a final dilution of approximately 1.5% sodium citrate, 0.5% phenol and 10% glycerin in the finished product. The serum thus preserved keeps well for several months. *Each serum should be handled separately to avoid accidental mixing or confusion of the two groups.*

Sources of error.—Ottenberg cites the sources of error as being: (1) Deteriorated sera. This possibility suggests two essential precautions which can be demanded in all authentic work: (*a*) Every test must be done in duplicate with two different sera of each test group (II and III). (*b*) Test sera must be shown to be active at the time of the tests. This must be controlled by using them against known Group II and Group III cells within at most a few days of the tests. (2) Sera originally weak. (3) Haemolysis. When haemolysis occurs, agglutination can nevertheless be demonstrated, either by first inactivating the serum and washing the red cells or by keeping the test from the start at ice box temperature. The chief danger is partial haemolysis combined with weak agglutination, in which case the two can obscure each other. (4) Incubation at 37°C. (5) Drying. (6) Settling of cells. (7) Use of microscope. (8) Too thick a cell emulsion. (9) Undeveloped group characters. (10) Autoagglutination.

Substitutes for Blood.—To replace blood lost, the best fluid is blood; but if a donor is not available, or if from a standpoint of military efficiency it seems unwise to solicit donors, recourse must be had to other fluids. Then too occasion will frequently arise for the employ-

ment of other fluids intravenously for the purposes of medication or nutrition.

Fluids to be suited to such employment should possess certain general characteristics and properties. They should be sterile, and free from turbidity and sediment. Mellon and others, having noted that the pH of many solutions varies decidedly from that of the blood, report that they obtained practical freedom from "systemic reactions" by buffering solutions when possible to the pH of the blood with phosphates. (See p. 690.) Undoubtedly the prime requisite is that solutions used intravenously produce no untoward effects such as injury to cellular structures by reason especially of osmotic action, this innocuousness being secured by selection of constituents and by ensuring that they are present in proper molecular concentration. Considerable latitude is possible in the choice of fluids either to increase the volume of the blood or to serve as vehicles. Among those available are NaCl solutions, Locke's solution, Ringer's solution and numerous modifications of these.

It would appear that blood bulk is the first indication to be met in cases of shock and after acute haemorrhage since the erythrocytes may be temporarily reduced to 50% without serious results provided there is made available sufficient fluid to mobilize them. Unfortunately, however, the effect of salt solutions in combating anoxaemia is temporary in that they add to the blood no oxygen-carrying substance to replace that lost, and their action in maintaining the circulation of the remaining erythrocytes is brief, the fluid rapidly leaving the blood vessels. In order to retain the fluid within the vessels for a longer time, various colloids have been added to saline solutions, and they may serve to tide a patient over a crisis. Bayliss has advocated the use of acacia for this purpose and undoubtedly it possesses certain advantages over gelatin, but the use of acacia solutions has recently been subjected to severe criticism in several respects on experimental grounds despite the excellent clinical results that have been reported.

To prepare an acacia solution for intravenous use, grind 60 grams of the *best* acacia (colorless lumps) to a powder, suspend it in 300 cc. normal saline, and stir until largely dissolved. Let stand in refrigerator over night. (If desired, one may here add 500 cc. of a strained 40% solution of pure, anhydrous glucose in normal saline.) Add sufficient normal saline to make one liter. Heat for thirty minutes in a boiling water bath, stirring frequently. Restore volume with distilled water. Titrate and neutralize with 10% Na_2CO_3. Autoclave for fifteen minutes at 15 pounds pressure. Filter through cotton, and then by suction through alternate layers of linen and hard filter paper (such as Whatman No. 50), two of each. Distribute in 300 cc. lots in Erlenmeyer flasks, autoclave *exactly as before* (variation will produce precipitate), and, if clear and free from sediment, store in refrigerator for use. The solution will keep a long time.

CHAPTER XIV

NORMAL AND PATHOLOGICAL BLOOD

In considering what may be termed normal blood, it must be borne in mind that the normal varies for men, women, and children:

	Hb.	Red Cells	Leukocytes
Men,	90 to 110%,	5 to 5½ million,	7500
Women,	80 to 100%,	4½ to 5 million,	7500
Children,	70 to 80%,	4½ to 5 million,	9000

Emerson in a study of the blood of 176 men students from 20 to 25 years of age reports the following findings: Means of reds, 5,000,000 (extremes 4,500,000 and 6,700,000); fourteen (8%) were below 5,000,000 and fifteen (8.5%) above 6,000,000. Mean of leukocytes, 7500 (52 cases). Mean of haemoglobin, 14.5 Gm. (Miescher); 92% (Fleischl); 87% (Dare); 92% (Gowers). Women medical students, same age limits, 16 cases: Mean of reds 4,800,000; of leukocytes, 8000; of haemoglobin, 11 Gm. (Miescher); 85% (Fleischl), 87% (Dare); 82% (Gowers).

Color Index

This is obtained by dividing the percentage of the haemoglobin by the percentage of red cells, 5,000,000 red cells being considered as 100%.

To obtain the percentage of red cells it is only necessary to multiply the two extreme figures to the left by two. Thus if a count showed the presence of 1,700,000 red cells, the percentage would be 34 ($17 \times 2 = 34$). If the Hb. percentage in this case were 50, then the color index would be $50 \div 34$, or 1.4.

In normal blood the color index is, approximately, 0.9.

In anaemias we have three types of color index: 1. The pernicious anaemia type, which is above 1. Here we have a greater reduction in red cells than we have of the haemoglobin content of each cell. For example, in a case of pernicious anaemia we have 2,000,000 red cells (40%) and 60% of haemoglobin, $60 \div 40 = 1.5$. 2. The normal type, when both red cells and haemoglobin are proportionally decreased, as in anaemia following haemorrhage. 3. The chlorotic type. Here there is a great decrease in haemoglobin percentage, but only a moderate decrease in the number of red cells. Hence the color index is only a fraction of 1. For example, in a case of chlorosis we have 40% of haemoglobin and 4,000,000 red cells, $40 \div 80 = 0.5$.

One can judge fairly well the approximate color index by noting the character of the staining of the red cells. This is faint in bloods of low color index and deeper than normal in cells in a case with high color index.

Red Cells

In considering the corpuscular richness of a specimen of blood, it must be remembered that this does not necessarily bear any relation to the quantity of blood in the body. Thus, a more or less bloodless-looking individual, the total quantity of whose blood is greatly reduced, may, notwithstanding, give a normal red count. In examining a specimen of peripheral blood we get a qualitative, not a quantitative result.

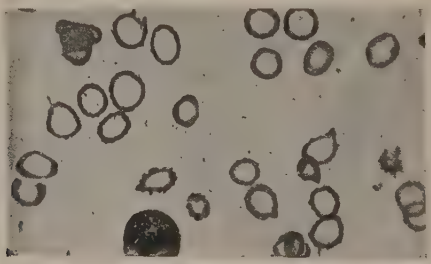


Fig. 84.—Red blood corpuscles showing deficient haemoglobin (achromia). From a well marked case of chlorosis. Wright's stain (×750.) (*J. C. Todd "Clinical Diagnosis."*)

Normally, we have an increase in red cells in those living at high altitudes. An altitude of 2000 feet may increase the red count about 200,000, and a height of 6000 feet about 500,000. Profuse sweats and diarrhoeas also increase the red count. Pathologically, in chronic polycythaemia with cyanosis and splenic enlargement, we have a red count of about 10,000,000. In cyanosis from heart disease, etc., and in Addison's disease, there is also an increase in red cells.

The normal red cell or erythrocyte measures about 7.5μ in diameter and 2μ in thickness. It is nonnucleated and normally stains with acid dyes, taking the pink of eosin or the orange of orange G. If larger, 10 to 20μ, it is called a macrocyte; if smaller, 3 to 6μ, a microcyte. It is usually stated that the life of a red cell after leaving the bone marrow is about thirty days. On this basis $\frac{1}{30}$ of the total number of red cells are destroyed each day. The old and damaged red cells are destroyed by the macrophages of various internal organs, chiefly the spleen. The bone marrow normally or even after considerable loss of blood pours out normal type red cells. If the loss of blood be great or continuous then various types of immature red cells are given off. Such cells show regenerative power of the bone marrow. Should these pathological cells not appear in the blood of a case of severe anaemia, showing lack of regenerative power of the bone marrow, we apply the term *aplastic* to such an anaemia.

Anisocytosis is a term applied to a condition where marked variation in size of the red cells occurs.

Macrocytes (10–18μ) are rather indicative of severe forms of anaemia, the *microcytes* (1–6μ), of less grave types. When the red cell is distorted in shape, it is called a *poikilocyte*. Care must be exercised that distorted shapes are not due to faulty technique. Crenation and vacuolation of red cells are marked in poorly prepared specimens.

In addition to variation in size and shape, we have also pathological variation in staining affinities.

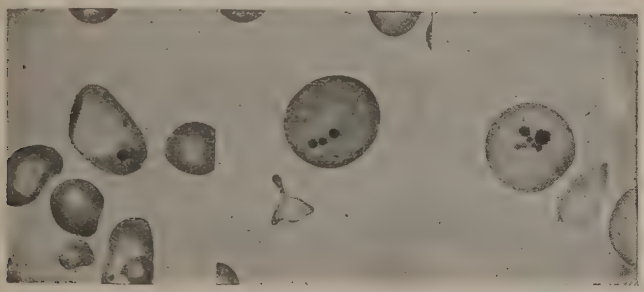


FIG. 85.—Nuclear particles or Howell-Jolly bodies in red corpuscles. From a case of pernicious anaemia. Wright's stain (×1000). (*J. C. Todd "Clinical Diagnosis."*)

Achromia.—This is characterized by pallor of the central portion of the stained red cell. It is indicated also by a central vacuolation in fresh blood cells which is apt to deceive one in the anaemic blood of malaria.

Polychromatophilia.—This shows itself by red cells taking a brownish to a dirty blue tint, as is frequently seen in immature red cells, especially nucleated ones.

Granular basophilic degeneration (also termed punctate basophilia and stippling) refers to the presence of blue dots in the pink background of stained red cells. It is found in many severe anaemias, as pernicious anaemia, the leukaemias, malarial cachexia, etc. It is very characteristic of lead poisoning.

This punctate basophilia (Grawitz granules) often coexists with polychromatophilia (polychromasia). It is not determined whether these granules mark degenerated cells or immature ones.

Nuclear Fragments.—Bloods showing nucleated reds may have red cells which fail to show a definite nucleus but possess irregular fragments of nuclear material.

Howell's Bodies.—These bodies stain a pinkish or bluish color with Giemsa's stain. They are larger than the Grawitz granules and are quite round and well differentiated from the red cell.

There are rarely more than two present in a cell. They are most often noted in blood showing punctate basophilia.

Cabot's Rings.—These are lines in loops or figures-of-eight which appear in Giemsa-stained blood of severe anaemias.

Reticulated Red Cells.—These are best demonstrated by vital staining which brings out a basophilic reticulum that differentiates such cells from normal red cells.

In normal human blood these cells are very rare, not more than 1 to 500 but in rabbits' blood they are as 1 to 50. In many anaemias they reach a percentage of 1 or 2% and in haemolytic jaundice may amount to 15% of the red cells. These bodies indicate increased marrow activity, which is shown also by appearance of nucleated reds, Howell bodies and Cabot rings.

Chromatin Dust.—This term is used to describe single or double small red granules usually seen at the periphery of the red cell. They are smaller than Howell's bodies.

Erythroblasts.—The nucleated red cell, while normal for the marrow, is always pathological for the blood of the peripheral circulation.

Normoblasts have the diameter of a normal red cell. The nucleus is round and stains intensely with basic dyes, often appearing almost black. Another characteristic is that it frequently appears as if embedded in the red cell. In normoblasts the red cell proper stains usually normally, but may show polychromatophilia in some instances. Some give the term microblast to smaller nucleated forms. The megaloblasts not only have a greater diameter than the normoblast, but the nucleus is poor in chromatin, stains less intensely and is less distinctly outlined. Instead of being round, the nucleus is irregular and may be trefoil in shape. The cytoplasm surrounding the nucleus shows polychromatophilia. This, contrasted with the pure blue of the lymphocytes, should differentiate. Megaloblasts belong to the bone marrow of early embryonic life and occur in the bone marrow of adults in very small numbers.

Normoblasts are found in secondary anaemias, and especially in myelogenous leukaemia. Megaloblasts are peculiarly characteristic of pernicious anaemia. Enormous megaloblasts are sometimes termed gigantoblasts.

At times, in the anaemias, there is a great temporary activity of the bone marrow and the blood of the peripheral circulation shows great numbers of erythroblasts. This is called a "blood crisis."

The term *leukanaemia* has been employed to describe conditions which partake of the characteristics of pernicious anaemia and leukaemia.

White Cells

Owing to the conflicting views as to origin, nature, and functions of the various leukocytes, their classification is in a state of confusion. It would seem that if all laboratory workers would agree upon some single method of recording differential counts it would be advantageous.

As regards the appearance of the cells, this of course, varies with the stain used, and it requires considerable experience for an individual to be able positively to recognize the difference between a lymphocyte and a large mononuclear when one specimen is stained with a Romanowsky stain, another with Ehrlich's triacid, and a third with haematoxylin and eosin. The difficulty, of course, is intensified when different persons adhere to the method of staining which they prefer and are at a loss to appreciate differences which are brought out by some other stain used by some other person. Even with the same stain used with different specimens of blood we find the staining characteristics of various leukocytes imperceptibly merging the one into the other, so that at times it is impossible for an observer, even with his own standard of differentiation, to be sure whether he is dealing with a lymphocyte or a large mononuclear. The difficulty is even greater when we deal with Türck's irritation forms and with myelocytes.

Without going into the various granule stainings so thoroughly brought out by Ehrlich, we shall immediately take up the question of a practical classification for use in making a differential count.

As the Romanowsky method of staining (Wright, Leishman, or Giemsa) gives us information not yielded by either haematoxylin and eosin or the triacid, the points of differentiation to be referred to in that which follows is with blood Romanowsky-stained.

Such staining brings out selective staining affinities which enable us to designate different leukocytes, or their several parts, as basophilic, acidophilic, or—in case of those that may be said to be on the borderline—neutrophilic.

With Wright's stain we have the eosinophile or oxyphile affinity of the granules of eosinophiles for acid dyes, in this case eosin. The nuclei and basophile granules have affinities in greater or less degree for basic stains (the blue and the violet shading resulting from methylene blue as modified by polychroming). With the granules in the cytoplasm of the polymorphonuclears and neutrophilic myelocytes, and to a less extent in the transitional, we have a staining which merges into a yellowish-

red on the one extreme and into a lilac on the other. As a standard, neutrophilic granules should be a mean of these extremes. With a Wright stain showing acid tendencies one may count polymorphonuclear neutrophiles as eosinophiles unless one notes the smaller size of the granules.

Not only by reason of the authority of Ehrlich, but because such a division gives all variations, which can then be combined by one preferring a simpler classification, it would seem proper to divide the normal leukocytes into hyaline and granular cells. Of the former we have the lymphocytes, the large mononuclears and the transitionals; of the latter the polymorphonuclears, the eosinophiles and the mast cells (basophiles).

Hyaline Leukocytes

1. Lymphocytes.—As a rule, the cells of this type are about the diameter of a red cell. They have origin in bone marrow and possibly also in lymphadenoid tissue. The nucleus is generally quite round but may show one or more bulging processes.

The large lymphocytes are rare in the blood of adults but make up about 10% of the leukocytes of young children. In making a count it is best to group large and small lymphocytes under one heading but for distinction we may divide them into:

(*a*) *Small lymphocytes.*—These are small round cells about the size of a red corpuscle with a large centrally placed, deeply violet staining nucleus and a narrow zone of cytoplasm. This cytoplasm may not be more than a mere crescentic fringe. This is the type of lymphocyte which makes up the greater proportion of the leukocytes in chronic lymphatic leukaemia. At times these cells seem to be composed of nucleus alone.

This nucleus contains one or two nucleoli. These cells are often called small mononuclears. In infants they make up more than 50% of the leukocyte percentage.

(*b*) *Large lymphocytes.*—These are of the same type as small lymphocytes, but possess more cytoplasm. The nucleus, while round and taking a fairly deep rich violet stain, does not stain so deeply as the nucleus of the small lymphocytes.

The cytoplasm is a clear, translucent, pure blue. It may contain pinkish granules known as azur granules, but these are of rather large size and do not mar the glass-like appearance. They are from 12 to 15μ in diameter and are common in children. In the acute lymphatic leukaemias they at times predominate.

These cells are similar to the cells of the germinal centers of the lymphatic glands.

2. Large Mononuclears.—These are large round or oval cells with a nucleus which has lost the richness of violet staining of the lymphocyte nucleus. The nucleus is furthermore frequently irregular in outline or may show the commencing indentation of the transitional nucleus.

There is not that sharp distinction between nucleus and cytoplasm that exists in the lymphocytes. The cytoplasm of the large mononuclear gives the impression of opacity, as if it were frosted glass instead of clear glass. This is due to very fine neutrophile granules rather like the larger neutrophilic granules of polynuclears. It is principally by the washed-out staining of the nucleus and the opaque lilac of the cytoplasm that we differentiate them from the lymphocytes. They greatly resemble Türck's irritation forms or plasma cells and may be confused with myelocytes.

These cells are considered to have great phagocytic activity and tend to migrate from the blood stream and become transformed into other forms. The term "monocyte" is frequently applied to these cells. They are assumed by some observers to take their origin in the reticulo-endothelial system, and there is considerable evidence in support of the view that these phagocytic cells include the endothelial cells, reticular cells of the spleen and lymph tissues and the reticulo-endothelium of the spleen, lymph and marrow sinuses, of the lung, suprarenal and hypophysial capillaries, and the Kupffer cells of the liver. The terms, "clasmatocytes," "macrophages," and "fibrocytes" have been applied to these mononuclear cells.

3. Transitionals.—These appear as but a later stage in the decay of the large mononuclears; the nucleus is more indented, frequently horseshoe-shaped, and has a washed-out violet shade of less intensity than that of the large mononuclears. These are the cells so often disrupted in smears. A good name for the fragmented cell smears is "smudges." The old view that the transitional was the precursor of the polymorphonuclear has few advocates at the present time.

While it may be convenient to consider the several hyaline cells as representing different stages in development, yet from a standpoint of immunity this is untenable. The large mononuclears and transitionals are the cells in which we find certain animal cells and pigment phagocytized, as is the case in malaria. These cells are the macrophages of Metchnikoff and are probably derived from the bone marrow.

In the tropics one of the most important points in a differential count is the matter of an increase in the large mononuclears and transitionals, both of which seem to respond to the same stimulus, which is most commonly malaria but may also be other protozoal infections.

From a practical standpoint I always group them together and as a matter of fact it is difficult to separate a large mononuclear showing considerable irregularity of nucleus from a transitional with less marked nuclear indentation.

Origin of Hyaline Leukocytes.—The lymphocytes take origin from bone marrow and the lymphoid tissue, and very probably the large lymphocyte is a younger, more immature cell than the small lymphocyte.

Ehrlich and Naegeli regard the large mononuclears as of myeloid origin while Pappenheim considers them to belong to the group of lymphocytes.

A normal percentage of large mononuclears and transitionals combined should not exceed about 4%.

Granule-containing Leukocytes

In addition to the series of leukocytes just considered we have present normally in the blood three types of granular cells distinguished according to the staining affinity of their granules. These are:

1. **Polymorphonuclear Leukocytes.**—This cell normally constitutes the greater proportion of the leukocytes. It is an amoeboid, actively phagocytic cell, about 10 to 12μ in diameter, and is the microphage of Metchnikoff.

Bacteria are actively phagocytized by this cell, and it is the cell concerned in determining the opsonic power of blood to various bacteria. It has fine lilac granules which are termed neutrophilic (epsilon granules). The single nucleus, rich in chromatin, is lobose like the kernel of an English walnut; frequently it resembles the letter z. These cells are derived from the neutrophilic myelocytes of the bone marrow. It is in these cells that the glycogen, or iodophile, granules, appear in certain suppurative conditions.

2. **Eosinophile Leukocytes.**—These are very striking cells with coarse granules staining brilliantly pink—the eosinophile, oxyphile, or acidophile granules (alpha granules of Ehrlich). The cells are a little larger than the polymorphonuclears.

The normal eosinophile is to be distinguished from the eosinophilic myelocyte by possessing two distinct lobes in the nucleus, or, at times, three nuclei; while the nucleus of the myelocyte is round. The eosinophile is the cell so frequently increased in infections by intestinal parasites.

We have frequently noted the complete absence of eosinophiles in smears taken from patients with suppurative appendicitis and acute infectious diseases, such as pneumonia, and their reappearance following recovery from these infections. Simon regards this phenomenon of considerable diagnostic importance and believes that it has not received the attention it deserves.

3. **Mast Cells.**—These also have coarse granules, but they stain a deep violet blue. Hence they are basophile granules (gamma granules).

In fresh blood these granules do not show up very well, being thus distinguishable from the highly refractile granules of the eosinophile. The trilobed nucleus stains less intensely than the granules. As a rule, the mast cell is about the size of a polymorphonuclear.

In a differential count of normal blood we find about the following percentages:

Polymorphonuclears,	65 to 70%,	about 5000 per cu. mm.
Small lymphocytes,	20 to 30%,	about 1500 per cu. mm.
Large lymphocytes,	2 to 6%,	about 200 per cu. mm.
Large mononuclears,	1 to 2%,	about 100 per cu. mm.
Transitionals,	2 to 4%,	about 200 per cu. mm.
Eosinophiles,	1 to 2%,	about 100 per cu. mm.
Mast cells,	0.25 to 0.5%,	about 25 per cu. mm.

Note.—The usually accepted standard in a differential count is that of Ehrlich: Polymorphonuclears, 70–72%; eosinophiles, 2–4%; basophiles, 0.5%; lymphocytes, 22–25%; large mononuclears, 1%, and transitionals, 2–4%. Such a count is based on Ehrlich's tri-acid stain, with which the percentage of polymorphonuclears tends to be increased and that of lymphocytes decreased, as compared with results obtained with Wright's stain. As some modification of the Romanowsky stain is generally used at present, the normal percentage of polymorphonuclears should be placed at about 65 and that for lymphocytes 30. The cells which particularly tend to vary under various conditions such as altitude, digestion, exercise and local climatic influences are the lymphocytes which show increase at the expense of the polymorphonuclears. The other leukocytes are less affected. One should always keep in mind the relative lymphocytosis of children but even in adults lymphocyte counts over 30% are not to be regarded as pathological. As to a standard of total leukocyte count we should not regard counts between 7000 and 10,000 as other than normal ranges.

Blood cell calculator.—A mechanical calculator on the principle of the adding machine has been devised to facilitate blood cell counting. Tabulation of cells can be made by pressing keys corresponding to various types of cells without removing eyes from the microscope.

Differential Count

In making a differential count I would recommend the following from the directions of Schilling-Torgau.

It will be remembered that considerable interest was raised a few years ago in what was termed the Arneth index. In this the more normal, more mature, better resisting polymorphonuclears were considered to have three or four lobes to the nuclear structure, occasionally even five. The immature cells had only one or at most two lobes to the nucleus. The index was obtained by adding the percentages of cells showing one and two lobes to one-half the percentage of those with three lobes. As will be understood a high percentage of these immature cells was unfavorable in prognosis. These cells are graded from left to right I, II, III, IV, V, as

to separate masses in the nucleus, so that when the percentage is shoved or displaced to the left it indicates an increase in the immature cells. The test was used particularly as a prognostic indication in tuberculosis and various pyogenic infections.

Schilling-Torgau divides his polymorphonuclears into: (1) The myelocyte which is always of course a pathological cell; (2) the immature form polymorphonuclear. In this there is a close resemblance to the neutrophile myelocyte but there is a nuclear indentation instead of the round nucleus of the myelocyte. It is this cell which often puzzles us as to whether to regard it as a true myelocyte. It is the metamyelocyte of many authorities. (3) Between the mature or segmented polymorphonuclear and the immature one or metamyelocyte we have what may be designated the band-form nucleated one. These show the type of nucleus which one is familiar with in the nucleus of the transitional. (4) The mature, multilobed or segmented nucleus of the typical polymorphonuclear.

In the differential count he not only divides up the polymorphonuclears but makes no separation of small from large lymphocytes. Although I have always divided lymphocytes into large and small ones I believe it unnecessary and impractical and shall henceforth group all such cells in one grouping. The statement that large mononuclears and transitionals are cells of a similar origin, type and significance has always been my idea.

Scheme of Schilling-Torgau

Type of Cell		Normal Percentage	Percentage Moderate Sepsis (W. C. 14,000)
1. Mast cells.		1	1.0
2. Eosinophiles.		3	1.5
3. Neutrophiles.	*a.* myelocytes.	0	0.5
	b. immature forms (metamyelocytes).	0	5.0
	c. band-form (Stabkernige).	4	13.5
	d. multilobed (Segmentkernige).	63	64.0
4. Lymphocytes.		23	10.5
5. Larger mononuclears and transitionals.		6	4.0

Pathological Leukocytes

The leukocytes which are found in the peripheral circulation only in pathological conditions are:

1. Neutrophilic Myelocytes.—The common type is a large cell with a large centrally placed, feebly staining nucleus.

This may be recognized by the difficulty of distinguishing the nucleus from the cytoplasm, there being no sharp line separating these parts of the cell. They imperceptibly merge into one another. They differ from a large mononuclear in that the cytoplasm is distinctly dotted with neutrophile granules and that we cannot make out a distinct line of separation between slightly irregular or indented nucleus and the surrounding slightly neutrophilic cytoplasm. Cornil has described a very large myelocyte with eccentrically placed nucleus and neutrophilic granules.

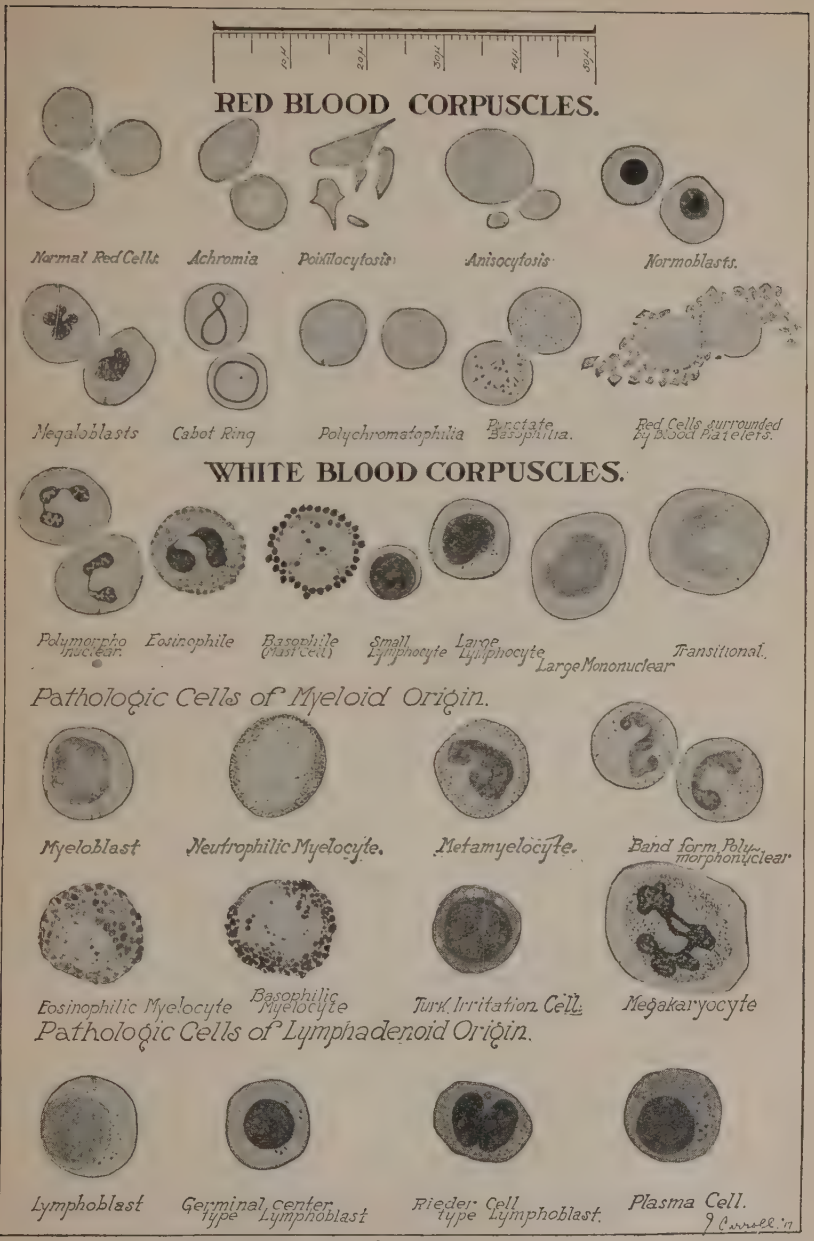


FIG. 86.—Normal and pathological blood cells.

Myelocytes are at times found with both basophilic and neutrophilic granules, and a single cell may rarely have all three kinds of granules—acidophile, basophile, and neutrophile.

2. Eosinophilic Myelocytes.—These can be distinguished from normal eosinophiles by their possessing a single round nucleus, not bilobed. These myelocytes may be as large as a normal eosinophile, but frequently are no larger than a red cell.

The neutrophilic myelocyte is characteristic of spleno-myelogenous leukaemia, the eosinophile one of myelogenic leukaemia. The occurrence of an occasional neutrophilic myelocyte is frequently noted in conditions having a leukocytosis. In diphtheria their presence in numbers is of bad prognostic import. Myelocytes are of diagnostic importance in metastases of malignant tumors.

3. The Irritation Cell of Türck.—This cell has a faintly staining, eccentrically placed nucleus, and a dark opaque blue, frequently vacuolated, cytoplasm. They are usually recorded as large mononuclears. Türck supposed them to appear in the circulation as the result of bone marrow irritation.

4. Myeloblasts.—These cells are found in myeloid leukaemia and though often mistaken for lymphocytes they are of marrow origin. They are the lymphoid cells of the marrow and are the parent cells of myelocytes.

The nucleus stains more intensely than that of the large mononuclear and the cytoplasm is more deeply blue stained than that of the large lymphocyte. They also show three or four nucleoli in the nucleus. They show peroxidase granule staining which the lymphadenoid cells never give. See Goodpasture's stain. There is no perinuclear clear zone and no azur granules in cytoplasm as have the pathological large lymphocytes.

Pyronin-methyl green staining is best for demonstrating the nuclei.

5. Lymphoblasts.—These are, as a rule, much larger than normal large lymphocytes and show heavy staining of both nucleus and cytoplasm. The nuclei often show the appearance of division into two or more lobes, thus showing the characteristics of Rieder cells.

They may be confused with large mononuclears but are considered to be derived from the germinal centers of various lymphoid tissues. They are found in leukaemic and pseudo-leukaemic conditions. They never show peroxidase granules. This characteristic together with sharper outlining of the nucleus differentiates the pathological lymphocytes (*lymphoblasts*) from myeloblasts.

6. Megakaryocytes.—These are the giant cells of the bone marrow and are but rarely found in the blood. The nucleus is gnarled.

7. Plasma Cells.—These, very rarely found in the blood, resemble lymphocytes but have an eccentric nucleus of wheel-spoke appearance. Some authorities designate Türck cells plasma cells.

These plasma cells are of lymphadenoid and not of myeloid origin as are Türck cells.

Blood Platelets

These are normally present in blood in the number of about 350,000 per cubic millimeter. They disintegrate very quickly after the blood is withdrawn. Wright has demonstrated that they are pinched-off projections of giant cells of the bone marrow. They consist only of protoplasm, no nuclear material. They do not contain haemoglobin. In conditions where giant cells are less abundant in the bone marrow, as in pernicious anaemia, the blood platelets are less abundant. In myelogenous leukaemia they are very abundant. They vary in size from 2 to 5μ according as a larger or smaller pseudopod of a giant cell has been broken off. Stained with Wright's stain, they are more purplish than blue and show thread-like projections. They are often mistaken for the protozoal causes of various diseases. Especially are they confused with malarial parasites when lying on a red cell. The blood plate has no brick-red chromatic material; it is purplish rather than blue, and has no pigment grains. It is advisable to compare these isolated blood plates with the larger or smaller aggregations scattered about the smears. In this way their true character becomes apparent. In addition to blood platelets, which are visible in fresh blood only when a fixative is used, we have other confusing bodies such as pinched-off fragments of red cells. The number of blood platelets is readily approximately estimated in a well stained Romanowsky preparation. Then too the variations in size show up well. One should always have his own mental picture of a normal blood platelet distribution. The Wright and Kinnicutt method is the standard one for their estimation. In this the blood is drawn up to 1 and a diluting fluid consisting of 2 parts of 1 to 300 brilliant cresyl blue and 3 parts of potassium cyanide (1 to 1400) is drawn up to 100. Blood platelets are increased in leukaemias and following haemorrhages as well as in trichinosis. In pernicious anaemia and severe infectious diseases they are diminished. In aplastic anaemia and in purpura haemorrhagica they are very scarce.

Fonio's method of platelet counting is as follows. The finger tip is cleansed with alcohol; then a drop of 14% magnesium sulphate solution is placed on it. Puncture is made through the drop which causes the drop of blood obtained to mix with the solution. Smear is then made and stained with Wright's or Giemsa's. Platelets appear isolated; both red cells and platelets are counted. 1000 red cells are counted and the number of red cells per cubic millimeter determined. It is simple to calculate the number of platelets. There is normally one platelet to fifteen or twenty erythrocytes.

The *haemokonia of Müller* are small, highly refractile bodies showing active oscillatory movement. They are supposed to be cast-off granules of eosinophiles or other leukocytes, or possibly derived from nuclei. As this blood dust or haemokonia is found in a marked degree in lipaemia it may be that the particles are fat. It is

interesting that this lipaemia is absent after the taking of large quantities of fat in cases with serious pancreatic trouble. The serum of a normal individual is rather turbid after slight indulgence in butter.

Leukopenia

This is a term used to designate a reduction in the normal number of leukocytes. A leukocyte count of 5000 would represent a slight leukopenia; one of 2000, a marked leukopenia. In the later stages of typhoid, and in acute miliary tuberculosis, we expect a moderate leukopenia. Glandular tuberculosis may give a very marked leukopenia, even under 1000. Tuberculous peritonitis will show moderate leukopenia or a normal count.

The leukopenia of typhoid is moderate and at times preceded in the first few days by a moderate neutrophile leukocytosis. Later on we have a decided increase in the lymphocytes. A marked diminution or absence of eosinophiles is so characteristic that any increase in eosinophilic percentage negatives a diagnosis of typhoid. During convalescence there may appear an increase in eosinophiles.

Paratyphoid gives a similar blood picture. Influenza is a disease which shows a reduced white count and in the post-influenzal pneumonias of the recent epidemic very low white counts were common, often running below 3000.

Chronic alcoholism and chronic arsenic poisoning cause a reduction in the number of the white cells. We have observed a patient in whom the white cell count fell to 800 following prolonged administration of arsphenamine. Pernicious anaemia, especially the aplastic type, shows a marked leukopenia, as is also the case with Banti's disease. Two tropical diseases, kala-azar and dengue, show a marked leukopenia, the counts often being below 2500. During the apyrexial period of malaria we may have a white count of 5000.

It has been claimed that a leukopenia with a coincident marked reduction in the lymphocytes is characteristic of measles and that this occurs several days before the Koplik spots appear.

Kocher notes that in exophthalmic goiter the leukocyte count is considerably diminished and that the polymorphonuclears are not much more than one-half the usual percentage while the percentage of the lymphocytes is almost double the normal.

X-ray treatment tends to destroy leukocytes in the exposed region, especially polymorphonuclears. The small lymphocytes are least affected.

Eosinophilia

Where the eosinophiles are increased to 5%, we have a moderate eosinophilia, but it must be remembered that the proportion of eosinophiles in the blood of children is greater than in that of adults. In

some cases of infection with intestinal parasites, especially hookworms, but also with other parasites, as round and whip-worms, we may have an eosinophilia of 30 to 50%. In Guam, among the natives, it is difficult to find an eosinophile count under 15%. The eosinophilia tends to disappear when the anaemia becomes very severe.

Echinococcus infection has an eosinophilia which disappears when the cyst is removed. Continuance of the eosinophilia indicates that all cysts were not removed.

The eosinophilia of trichinosis is best known, and a combination of this blood finding with fever and marked pains of muscles, would justify the excision of a piece of muscle for examination for encysted embryos provided the period for getting embryos from centrifuged blood had elapsed.

Eosinophilia is marked in true or essential asthma, and absent in the secondary forms of the disease. Certain skin diseases, especially pemphigus, show eosinophilia. Blastomycoses are usually found to show eosinophile increase.

Eczema and psoriasis are not apt to give more than 3 or 4% eosinophiles. A rather high degree of eosinophilia is found in mycosis fungoides. Scabies also gives an eosinophilia.

At the height of the disease there may be a rather marked eosinophilia in scarlet fever.

Increase of both eosinophiles and mast cells is found in myelogenous leukaemia.

An eosinophilia tends to appear following splenectomy. An increase of eosinophiles often precedes and accompanies a spontaneous remission in pernicious anaemia.

Hodgkin's disease and sarcomatosis may show a marked increase of eosinophiles. Mucous colitis and serum sickness may show an eosinophilia.

Following the administration of camphor we may have a rise of eosinophiles to approximately 10%.

Leukocytosis

It is to an increase in the polymorphonuclears that this term is usually applied, the term lymphocytosis or eosinophilia being employed where white cells of eosinophile or lymphocyte nature are increased. We have physiological leukocytosis in the latter weeks of pregnancy, also in the new-born, and in connection with digestion.

Pathological Leukocytosis. *Pneumonia.*—In this disease we have a leukocytosis of 20,000 to 30,000 or higher. The eosinophiles are usually absent. A normal leukocyte count in pneumonia makes a prognosis unfavorable.

The leukocyte count drops about the time of the crisis, and with the reappearance of eosinophiles is a favorable sign.

Toxaemic conditions as uraemia, diabetic coma and poisoning by CO_2 tend to show a leukocytosis.

Septic processes.—The leukocyte count is of great value, especially when we obtain a leukocytosis with 80 to 90% of polymorphonuclears, as in appendicitis, cholecystitis, or other suppurative conditions. A marked leukocytosis is of diagnostic importance in acute ulcerative endocarditis provided it is not fulminant in type.

According to Cabot, leukocytosis varies in infections as follows:

1. Severe infection—good resistance; early, marked and persistent leukocytosis.
2. Slight infection—slight resistance; leukocytosis present, but not marked.
3. In fulminating infections we may have no increase in whites, but a higher percentage of polymorphonuclears.
4. Slight infection and good resistance may not be productive of leukocytosis.

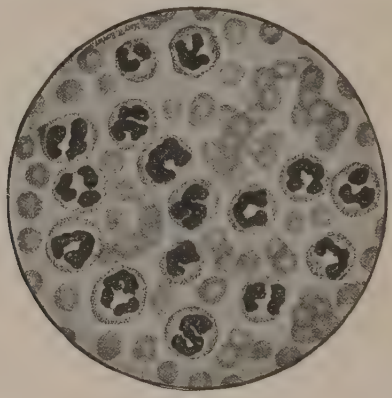


FIG. 87.—Leukocytosis (40,000); sixteen polymorphonuclears in field. (*Cabot.*)

It is in connection with the question of operation in appendicitis or similar conditions that the matter of a leukocyte count is of prime importance. If there be a leukocytosis but with less than 75% of polymorphonuclears it indicates an infection of little virulence or a walled-off process with an exacerbation. It is difficult to form an opinion when the polymorphonuclears are under 80%. Leukocytosis with polymorphonuclear percentage of 85 to 90 indicates immediate operation; percentages over 90 point to peritonitis and if with such percentages of polymorphonuclears there is absence of leukocytosis the prognosis is grave.

The blood of cases with malignant tumors tends to show a moderate keukocytosis except in epithelioma of the skin. When a cancer is ulcerating quite a high white count may be obtained.

Spirochaete fevers, as relapsing fever, may give a leukocytosis of from 12,000 to 17,000.

Smallpox, especially at time of pustulation, plague, scarlet fever, and liver abscess give a leukocytosis of from 12,000 to 15,000.

Smallpox often shows a very large percentage of very characteristic large mononuclears. In this disease a leukopenia precedes the leukocytosis.

The leukopenia and lymphocyte increase in measles are important points in differentiating it from scarlatina.

Influenza shows a leukopenia at first, then a leukocytosis and, following the fall in fever, a second lowering. In the recent epidemic there has seemed to be a rather constant leukopenia.

With meningitis, except in the tuberculous type, counts of 25,000 are not unusual. In abscess of the brain the white count rarely exceeds 15,000.

Poliomyelitis and polioencephalitis give a slight leukocytosis during the febrile accession.

Erysipelas and epidemic cerebrospinal meningitis also give a leukocytosis of from 15,000 to 20,000. In malignant diseases we sometimes have a moderate leukocytosis. Rogers states that in liver abscess, with a leukocytosis of 15,000 to 20,000, we have only about 75 to 77% of polymorphonuclears—there being also a moderate increase in the percentage of large mononuclears.

Drugs such as antipyrin may give a leukocytosis. The leukocyte increase of pilocarpine is rather a lymphocytosis. Cinnamate of soda, sodium nucleate, bacterin injections and turpentine have been used in kala-azar to increase leukocytes.

Lymphocytosis

Of course, the disease in which we have the most marked lymphocytosis is lymphatic leukaemia.

In glandular fever (Pfeiffer) there is often found a blood-picture easily mistaken for that of lymphatic leukaemia. See page 365.

The lymphocytosis of typhoid fever has been taken up under leukopenia.

Whooping-cough may give a lymphocytosis of 20,000 to 30,000.

Young children have normally an excessive proportion of lymphocytes even to a reversal of the polymorphonuclear-lymphocyte relation of adults. This is apt to be particularly marked in hereditary syphilis. Enlarged tonsils may give rise to a leukocytosis of 10,000 to 15,000 when more than 50% of the white cells will be lymphocytes. Rickets and scurvy give a lymphocytosis.

In pellagra there is a moderate lymphocytosis, averaging 34% in about a normal count.

Varicella and mumps may also give an increase in the percentage of lymphocytes.

Undulant fever is a disease which may show quite a lymphocyte increase, this going with a reduction in polymorphonuclears.

Increased Large Mononuclears

In tropical work we combine the large mononuclears and transitionals in a differential count. They are the phagocytes of animal cells or parasites. The disease in which their increase is best recognized is malaria and an increase to 15% where the blood shows moderate leukopenia is very significant. The melaniferous leukocytes of malaria are cells of this type.

Other protozoal infections, as kala-azar, trypanosomiasis, yellow fever and amoebiasis cause it. Filterable virus diseases may show a mononuclear increase; thus dengue gives an increase about the fifth or sixth day.

In Banti's disease there is an increase in cells of this type, and a transitional increase is reported for Hodgkin's disease.

Diseases with a Normal Total Leukocyte Count

Uncomplicated tuberculosis, influenza, undulant fever, measles, trypanosomiasis, malaria, syphilis, and chlorosis.

In malaria we have a leukocytosis at the time of the rigor, while during the apyrexial period there is a moderate leukopenia. In malaria we have a marked increase in the percentage of the large mononuclears and transitionals. These may form from 25% to 35% of the leukocytes. (When bearing particles of pigment they are known as melaniferous leukocytes—macrophages which have ingested malarial pigment.) In dengue, at the time of the terminal rash, we may have as great a percentage of large mononuclears. In this disease, however, we have a great diminution of polymorphonuclears from the start (25 to 40%). Instead of a large mononuclear we have at the onset a lymphocytic increase. There is an increase of large mononuclears in trypanosomiasis.

The white count is about normal in uncinariasis (Ashford's average was 7800). Some have reported a leukopenia in severe cases.

While eosinophilia is the most marked feature in hookworm disease yet in very severe cases it may be absent.

The Polycythaemias

Under this designation are grouped conditions with increase of red cells. There are such states which may be secondary to respiratory or cardiac conditions, and there is a special disease, erythraemia, which is characterized by hyperplasia of red cell-producing marrow. The secondary polycythaemia connected with pulmonary stenosis, termed morbus ceruleus, is well known; in mitral valve disease also there may be an increased red cell count which rarely exceeds 7 millions, and with

congenital heart disease we may have counts approximating 10 millions, although increased red cell count is not a constant accompaniment of this affection.

Erythaemia.—This disease is also known as Vaquez's disease or polycythaemia vera. The cause is unknown. The onset is insidious and the course chronic. Headache, dizziness and constipation are common. The patient shows a brick-red color especially of hands, face and mucous membranes, which is more marked in cold weather. The spleen is often enlarged and the blood pressure may be high. The viscosity of the blood is much increased so that it is difficult to make smears. The red cells run from 7 to 10 millions, with increase of haemoglobin percentage, but to a less extent than that of the red cells, so that the color index is below 1. There may be an occasional nucleated red. There is a moderate leukocytosis (polynuclear) and occasionally a myelocyte may be noted. There is a tendency to bleeding in this disease, especially of the gums.

The Primary Anaemias

Chlorosis.—In chlorosis it is the reduction of haemoglobin with the slight numerical variation from normal of the red cells that makes for a diagnosis. The color index is very low. There is nothing abnormal about the leukocytes. Microcytes may be present, and very occasionally a normoblast. Macrocytes and megaloblasts are absent. It is only in very severe cases that we see poikilocytes. At the present time this disease appears to be rather uncommon.

Blood of chlorotics is very pale and very fluid and coagulates rapidly; hence frequency of thrombosis. There is an increase in the blood platelets.

Spleen, liver and lymph glands as a rule are normal.

Simple Primary Anaemia.—This condition is not recognized by many authors but is a convenient term under which to group anaemias which are neither chlorosis, nor pernicious anaemia, and for which no assignable cause can be ascertained. In it the color index is about normal, there is no change in the leukocytes and cases go on to recovery.

Pernicious Anaemia (Addison-Biermer Anaemia).—In pernicious anaemia we obtain a very fluid and lightly colored drop of blood upon puncture. The viscosity is lowered and the coagulation time prolonged. The yellow marrow of the long bones is transformed into a soft, gelatinous, bright-red lymphoid tissue, smears from which may show great numbers of megaloblasts.

Areas of fatty degeneration are characteristic, especially the "tiger-lily" spots in the heart muscle. Fatty degeneration of liver and kidneys is also marked. Iron-containing pigment (haemosiderin) is found in the liver, spleen, and kidneys. Areas of degeneration in the spinal cord may account for nervous symptoms. The red

cells frequently fall below 2,000,000 with patients still ambulant. Cases have been reported with counts under 200,000. *The color index is high.* Macrocytes and megaloblasts are the most characteristic qualitative change in the red cells. Decrease in blood platelets is usually noted. With the high color index there is leukopenia. In my opinion a marked anisocytosis is more a feature of the disease than the presence of megaloblasts. Even during periods of remission a preponderance of macrocytes is usually found. Urobilinuria is marked.

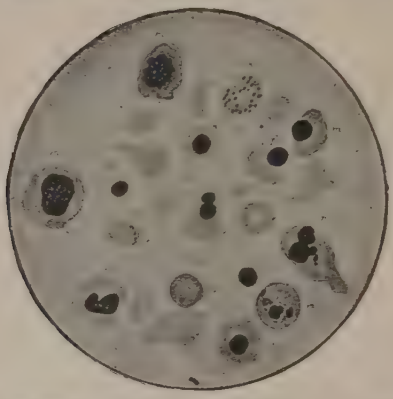


FIG. 88.—Pernicious anaemia. *M.m*, Megaloblasts; *n*, normoblast; *s*, stippling (punctate basophilia). (*Cabot.*)

Blood crises.—When the circulation is suddenly flooded with large numbers of nucleated reds such regenerative manifestation is called a "blood crisis." Megaloblastic crises may at certain times show enormous numbers of megaloblasts. Cases often present remissions in which no megaloblasts can be found. In such cases the presence of many macrocytes, especially if these are oval in form, should prevent an examiner from reporting against a pernicious anaemia previously diagnosed as such.

Poikilocytosis, polychromatophilia, and stippling are also features of the disease. Normoblasts are far less frequent than megaloblasts and there is usually a moderate lymphocyte increase with polynuclear decrease. Myelocytes may be present, but their precursors, the myeloblasts, are probably more frequently met with.

Symptoms.—The onset of the disease is insidious, the patient developing a lemon-yellow color and weakness. Many cases fail to show any signs of emaciation. There are often accessions of alimentary tract disorders and anorexia is usually marked. Functional heart murmurs, palpitation and dyspnoea are usually present. Petechial spots may occur but pigmentation is rare.

Cases of pernicious anaemia show remissions during which the patient is apparently on the road to recovery. Such improvements are only temporary. The remissions may last from two months to possibly three or four years.

In pernicious anaemia there is usually an absence of free HCl in the stomach contents, which causes it often to be confused with gastric cancer. Cases of chronic nephritis are also often much like pernicious anaemia; but most difficult of differentiation are affections of the spinal cord, such as tabes, etc., the neurological manifestations of pernicious anaemia causing the confusion. Retinal haemorrhages must be kept in mind. After repeated transfusions pernicious anaemia tends to assume the aplastic form.

Blood changes more or less like those of pernicious anaemia have at times been noted in children with tuberculosis of bovine nature. The human strain does not seem to produce such changes.

An acute disease showing a rapidly developing anaemia of the pernicious anaemia type is Oroya fever in which the bone marrow seems especially involved. See page 662.

Frequently in the anaemia of *Diphyllobothrium latum* we have a blood-picture of pernicious anaemia, supposed to be due to a toxin present in the heads of these tape-worms.

A severe anaemia in which the blood-picture is that of pernicious anaemia may accompany infections with the *Balantidium coli*.

Aplastic (Aregeneratory) Anaemia.—This is a very rare and rapidly fatal disease in which there is markedly diminished activity of the bone marrow so that the red cell blood findings which characterize the blood regeneration of pernicious anaemia are absent. Although the red cell count soon becomes very low there is neither anisocytosis nor nucleated reds. The blood platelets are almost absent. The color index is normal or reduced. Leukopenia rapidly develops at the expense of the myeloid cells so that the percentage of polymorphonuclears may soon reach figures approximating 10 to 15%. Purpura and mucous membrane haemorrhages soon show themselves. The urine is very light in color and shows no urobilin. The yellow of the bone marrow does not change to red, and smears from such marrow at autopsy show myeloblasts and rarity of nucleated reds.

Sickle Cell Anaemia.—Under this term have been described a number of cases of severe anaemia usually chronic in character and affecting negroes, characterized by the development of a crescentic distortion of the red blood cells.

The tendency to sickle cell formation is considered to rest on a hereditary predisposition and to be a familial trait. The red blood cell may not be distorted in fresh preparations of stained or wet smears, but this distortion may occur when sealed

wet smears are kept at room temperature for twenty-four hours. This would indicate that sickle cells are not produced as such by the erythropoietic tissue.

Hahn and Gillespie, in studying a patient with this type of anaemia, found that sickel cell formation is a reversible phenomenon depending on the oxygen tension of the medium in which the cells are suspended. In the presence of oxygen, the discoid form was stable; in its absence the distorted form was stable. They believed that sickle cell formation in susceptible individuals is induced or increased by anoxaemia.

This hereditary sickle cell trait is not a disease and persons showing this trait may be in excellent health. However, it predisposes those affected by it to haemolytic anaemia.

Secondary Anaemias

These are the anaemias which can be traced definitely to some disease not of the haemopoietic system.

There are two main groups—those following haemorrhage and those secondary to various diseases.

Acute Posthaemorrhagic Anaemias.—If the haemorrhage is sudden and great, the resulting condition is one of oligochromaemia—chlorotic in type. Normoblasts are usually found after the third day. Blood platelets are increased and coagulation time shortened.

In the regeneration the plasma restoration comes first, then the red cells and finally the haemoglobin. An increase in number of polymorphonuclears is always noted with an occasional myelocyte.

The low Hb. percentage is apt to continue for several weeks.

It is a question whether operations that are prolonged or require narcosis are justified where the amount of Hb. is under 40%. (According to Miculicz, 30% is the minimum.)

Chronic Posthaemorrhagic Anaemias.—Where the loss of blood is gradual, as in gastric cancer or severe haemorrhoids the picture may more nearly approach that of pernicious anaemia. Secondary anaemias usually show a moderate leukocytosis. In chronic nephritis and prolonged suppurative conditions normoblasts and macrocytes are rare—moderate poikilocytosis with the presence of many microcytes being the rule.

In fatal anaemia from chronic acetanilide poisoning, high color index, macrocytes and megaloblasts have been noted.

Secondary Anaemias from Disease.—In some secondary anaemias, as in syphilis, carcinoma, and tuberculosis, we have a chlorotic color index (chloro-anaemias).

In secondary anaemias, polychromatophilia, poikilocytosis, and punctate basophilia (stippling) may be present. This latter is very marked in lead poisoning, but in certain cases of malarial cachexia it may be equally prominent. The only form of nucleated red cell seen is the normoblast. If present, it is in very small numbers.

Megaloblasts are practically never seen, except in some of the very severe parasitic anaemias, as the broad Russian tape-worm infection. The red cells generally number between 2,000,000 and 4,000,000, thus differentiating chlorosis. The leukocytes are frequently increased to 15,000. In the anaemia of splenic anaemia there is a marked leukopenia. In anaemias from malignant tumors the color index is usually of the chlorotic type—the haemoglobin content of the red cells being more affected than the number. Normoblasts are usually present, and this finding may differentiate gastric cancer from ulcer. In malignant disease metastatic in the bone marrow, megaloblasts may be expected. Myelocytes and so-called tumor cells (large cells with faintly staining vacuolated nuclei and but little cytoplasm) may also be found. As a rule, there is a moderate leukocytosis in malignant disease. Eosinophiles may be largely increased in sarcoma.

Haemorrhagic Diseases

The haemorrhagic diseases include thrombocytopenia, haemophilia, and haemorrhagic disease of the newborn. Purpura may also accompany other diseases, such as the haemorrhagic forms of small-pox and scarlet fever. In fact, it may be a prominent manifestation in most of the acute infectious diseases. In scurvy, haemorrhages occur in the gums and around the hair follicles, but, if the disease is recognized and treatment instituted, the haemorrhages, as a rule, cease.

Thrombocytopenia.—This term is coming into more common use in place of purpura haemorrhagica and is a condition characterized by marked diminution in the number of blood platelets, spontaneous bleeding from mucous membrane, purpuric skin lesions, prolongation of the bleeding time, and normal coagulation rate of the blood, associated with failure of the clot to retract. The white cell count is often moderately increased, and the red cells show variations in size and shape. There may appear in the peripheral circulation immature and atypical cells of both the red and the white series.

The chief pathological feature of the disease is the marked reduction of blood platelets. The cause of this reduction is still somewhat obscure, and various theories have been advanced to account for this phenomenon.

It is now universally believed that blood platelets are independent elements of the blood, are derived from the megakaryocytes of the bone marrow, and reach the circulating blood by budding off from the mother cells. They possess ameboid movement. At times they are found in the spleen in great numbers, where they are normally destroyed. It has been demonstrated that when these elements are reduced to 50,000, even slight trauma will produce ecchymosis. Whether this reduction is due to toxins which affect the platelet centers in the bone marrow, or whether the platelets are destroyed in the circulation or in some other part of the body has not been absolutely proven. In view of the fact, however, that splenectomy so frequently results in a cure, it would seem reasonable to infer that platelets are destroyed at a more rapid rate than normal by the spleen itself.

The symptoms consist of haemorrhage from mucous membranes, varying from slight oozing to profuse bleeding. Bleeding from the gums is common, and there may also be epistaxis, haemorrhage from the uterus, the gastro-intestinal tract or the genito-urinary tract. There is sometimes bleeding into the internal organs, and even cerebral haemorrhage, which may result in death.

The cutaneous manifestations consist of purpuric spots, varying from a few petechia to extensive purpuric areas scattered over the body. These ecchymoses at first appear red in color, but within a few days fade and the color changes to bluish and yellowish-brown. There are frequently recurring crops of purpura.

The disease may occur in an acute form in which there is active bleeding accompanied by a mild fever and marked purpuric manifestations. The more common form is the chronic or intermittent type which may continue for years with recurring attacks of haemorrhage and petechial manifestations.

The disease must be differentiated from the other forms of purpura in which there is usually not such a marked reduction of blood platelets, and from the symptomatic purpura which occurs in other conditions, such as diphtheria, nephritis, tuberculosis and scurvy. The characteristics of this disease, described above, will practically always serve to differentiate it from other forms of purpura.

A considerable amount of evidence has been accumulated to show the value of splenectomy in this disease, particularly in its chronic form. In the acute form of the disease splenectomy is a more hazardous operation and appears to give less satisfactory results. In the chronic forms of thrombocytopenia splenectomy appears to offer the best chance for a cure and has been done in a sufficiently large number of cases to demonstrate its value.

The capillary resistance test of Hess is of value in this condition. A tourniquet is applied to the arm from one to four minutes, tight enough to obliterate the return circulation without obliterating the pulse. According to the degree of the haemorrhage diathesis, petechiae or even large ecchymoses appear on the forearm. According to the severity of the ecchymosis we may form some idea of the gravity of the condition. This test depends on the amount of diminution of platelets and the associated resistance of the capillary walls.

Schönlein's Disease.—This disease is characterized by a multiple arthritis accompanied by purpuric eruptions and is usually of shorter duration than thrombocytopenia. It is rarely fatal, and there may be recurrent attacks for several months. The purpuric spots usually appear on the lower extremities with a tendency to occur in groups. The joints are painful, swollen and tender, and the arthritic symptoms may subside before the purpura occurs. The disease resembles acute rheumatic fever, but appears to be a separate entity and is rarely complicated by endocarditis. There is not the marked reduction in blood platelets that occurs in thrombocytopenia.

Henoch's Purpura.—This disease is characterized by severe gastro-intestinal symptoms, abdominal pain, purpura, and bleeding from mucous membranes. There may be urticaria, erythema, and oedema. The severe abdominal symptoms may simulate appendicitis or other acute abdominal conditions. As a rule, the symptoms subside in a few days and the disease usually lasts less than a month. There may be arthritic symptoms and the disease may resemble Schönlein's disease, with the addition of severe abdominal symptoms. Blood in the stools often occurs, and may be considerable in amount.

Haemophilia.—Haemophilia may be defined as an hereditary disease occurring in males, but transmitted through the female, characterized by greatly prolonged coagulation time of the blood and a tendency to haemorrhage, which may be spontaneous and frequently follows slight trauma. Haemophiliacs, or "bleeders," as a rule, show no other abnormality except this tendency to haemorrhage. The blood picture does not show any characteristic change, and the red and white cells and blood platelets appear to be normal in all respects. The one positive finding in haemophilia is the prolonged coagulation time, which may be from twenty minutes to several hours. The degree of prolongation gives some idea of the severity of the disease. The reason for this prolonged coagulation time has not been satisfactorily explained. It is inferred that the deficiency in the elements of the blood which make for coagulation is a qualitative rather than a quantitative one. Fonio and also Minot and Lee believe that it is an hereditary defect in the blood platelets, which is characterized by their slow availability for the purpose of coagulation. The chief symptom of haemophilia is the tendency to bleed following a minor injury which, in a normal individual, would cause little or no haemorrhage. Haemophiliacs do not bleed from pin-pricks, and the vein or ear may be punctured to secure blood without injury, thus indicating that the bleeding time in haemophilia is normal. Prolonged and obstinate epistaxis is a common manifestation of the disease. Serious haemorrhage may follow the extraction of a tooth, and death has followed such a procedure. Haematuria, due to renal haemorrhage, which is difficult to allay, is occasionally encountered. There may be haemorrhages into the muscles following slight trauma, but purpuric spots do not occur. Joint symptoms are common in haemophilia, due to haemorrhage into the joint cavity. The diagnosis rests on the history of persistent bleeding following slight trauma, and an effort should always be made to secure a careful family history, which frequently throws light on this condition. The disease must be differentiated from thrombocytopenia, in which the bleeding time is prolonged, but the coagulation time normal; whereas, in haemophilia, the bleeding time is normal, but the coagulation time is greatly prolonged.

In haemorrhagic disease of the newborn, melaena is common, while it is rare in haemophilia. Bleeding from the umbilicus is uncommon in haemophilia, and the male haemophiliac, as a rule, does not have serious haemorrhages until he is older than the period at which haemorrhagic disease of the newborn occurs. The prophylactic treatment of haemophilia consists in guarding against even slight trauma, which may start the haemorrhage. When the haemorrhage occurs, various means, such as sutures, packs, local applications of coagulants, and transfusion are recommended.

Haemorrhagic Disease of the Newborn.—This is a condition characterized by spontaneous bleeding in infants shortly after birth. The haemorrhages are persistent, usually multiple, and usually occur during the first week of life. The underlying cause is still unknown and has been variously ascribed to some defect in the blood which delays the coagulation time, to abnormality in the blood-vessel walls, and to the presence of some obscure infection. The more generally accepted hypothesis is that the disease is due to a blood defect, which hypothesis has been further strengthened by the therapeutic effect of transfusion. In other respects these infants appear to be normal. The symptoms, as a rule, appear during the first week of life and are seldom observed after the twelfth day. The principal characteristic symptom is spontaneous and persistent haemorrhage, often multiple, observed in the

skin and subcutaneous tissues, and also from the mouth, nose, conjunctiva, and mucous surfaces. The meninges, pericardium, peritoneal cavity, kidneys, adrenals, thymus, lungs and liver may show haemorrhages. In fact, almost every tissue and organ of the body is susceptible to haemorrhage. There may be continuous oozing from the umbilicus, which is difficult to control by local measures. The stools often contain a large amount of blood. There is usually prostration with rapid pulse and an anaemia, dependent upon the severity of the haemorrhage. The blood picture shows no abnormality except the anaemia which results from the haemorrhage. The disease is self-limited and does not tend to recur.

The generally accepted treatment is the use of human blood by transfusion or subcutaneous injection. Transfusion appears to give the best results and frequently stops the bleeding completely.

The Leukaemias

It is in the leukaemias that we have the greatest increase in the number of white cells. These cases show more or less anaemia, but we may have cases of myelogenous leukaemia showing 250,000 leukocytes per cubic millimeter without particular change in the red cells. The more marked the red-cell change the more severe the condition.

There are two well defined types of leukaemia, viz. the lymphatic and the splenomyelogenous. It must be borne in mind, however, that while a greater change in the lymphatic glands may produce the lymphatic type, yet even in such cases we expect to find alteration in bone marrow and spleen; that is, there is a general involvement of the haemopoietic system in all leukaemias, the activity being most marked in spleen and bone marrow in certain cases and in lymphatic glands in others.

Myelogenous leukaemia is a very rare disease, about five times as rare as pernicious anaemia. Lymphoid leukaemia is still more rare.

Splenomyelogenous Leukaemia (Myeloid Leukaemia).—The differentiation of the blood-picture of this disease from leukocytosis does not depend on the number of leukocytes, but on the presence and large proportion of myelocytes.

We expect both neutrophilic and eosinophilic myelocytes in myeloid leukaemia—the proportion of these varies, but, as a rule, the neutrophilic one is the more common. The blood in advanced cases is milky and shows a most marked buffy coat. The marrow is largely replaced by a yellow pyoid material. The spleen may weigh 10 pounds.

Chronic myeloid leukaemia is insidious in onset, the first points to attract attention being the greatly enlarged spleen, pallor and prostration. Some cases have fever and night sweats which suggest tuberculosis.

The leukocyte count is on the average from 200,000 to 500,000. Cases are reported of more than 1,000,000 white cells. The neutrophilic myelocytes make up

about 30 to 40% of these and about equal in number are found the polymorphonuclears, while the percentage of the lymphocytes is decreased (2 to 5%) and normal eosinophiles, eosinophilic myelocytes, and large mononuclears make up the remaining percentages. Myeloblasts may be present as well as myelocytes and in exacerbations of the disease there may be many myeloblasts. We usually have great numbers of normoblasts. Megaloblasts may rarely be found. The red count is usually about 2,500,000 and the color index low. There is an *acute myeloid leukaemia* which runs a rapidly fatal course. The characteristic cell is the myeloblast. It is rare and is often confused with acute lymphatic leukaemia.

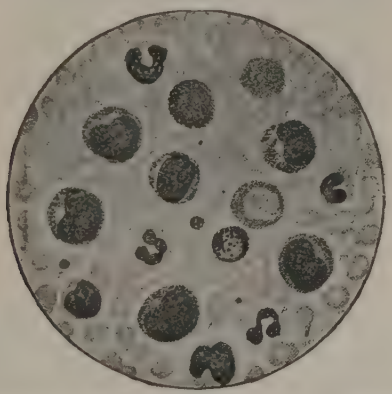


FIG. 89.—Myelogenous leukaemia. *m*, Myelocyte; *p*, polymorphonuclear; *b*, mast cell; *n*, normoblast. (*Cabot.*)

Lymphatic Leukaemia.—In this we have glandular enlargements, but not such large masses as in Hodgkin's disease. The red cells are usually reduced about one-half and the color index is a little below normal. Normoblasts are rarely found. Myelocytes, as a rule, are absent, but may amount to 5% of the leukocytes. The predominating leukocyte (75 to 98%) is the small lymphocyte. In acute lymphatic leukaemia the large lymphocytes and Rieder cells are the diagnostic ones.

These however are pathological and differ from the large lymphocyte in not having typical azur granules and in that the nucleus stains poorly and is often indented. The leukocyte count in chronic lymphatic leukaemia is never so great as in myeloid leukaemia, rarely exceeding 125,000.

Chronic lymphatic leukaemia has an insidious onset with increasing pallor and weakness. The glands in general tend to enlarge, most markedly, however, in the cervical region. This is one of the easiest of diseases to diagnose by a blood smear.

In *acute lymphatic leukaemia* there is a rapid course with fever and tendency to haemorrhages. The tonsils are often swollen and accentuate the usual stomatitis. At first the white count is about normal but in a week or so may run up to 150,000.

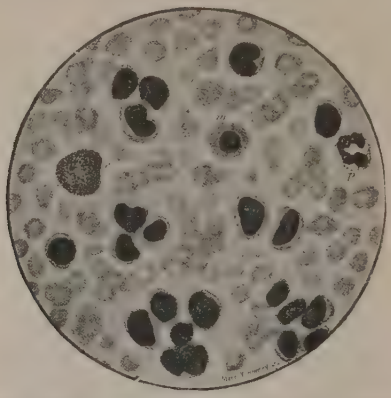


FIG. 90.—Lymphatic leukaemia. *p*, Polymorphonuclear; *m*, megaloblast; *e*, eosinophile. Twenty-one lymphocytes in this field. (*Cabot.*)

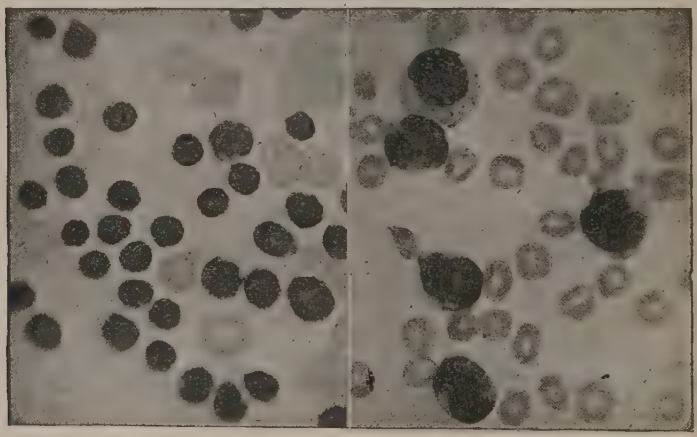


FIG. 91.—Blood in lymphatic leukaemia; ×700. On the left, chronic form of the disease; on the right, acute form (courtesy of Dr. W. P. Harlow). (*From "Clinical Diagnosis" by J. C. Todd.*)

THE PSEUDOLEUKAEMIAS

It is not that the diseases embraced under this designation have a relationship to one another but the term is used rather for the grouping of certain affections clinically suggesting leukaemia but not showing the leukaemia blood findings. Of course there are certain cases of aleukae-

mic leukaemia which fail to show white cell increase in part or throughout the course, but such cases are apt to show qualitative blood changes which can be recognized.

Glandular Fever.—Under this heading a disease has been described characterized by enlargement of the lymph nodes and spleen and accompanied by a moderate fever with symptoms of general malaise, weakness, and, frequently, sore throat. There is usually a moderate leukocytosis, and the appearance of the blood smear often resembles that of lymphatic leukaemia in that there are present many lymphoblasts of the germinal center type and the so-called Rieder cells, which may approach ninety per cent. of the total white cells. The term "infectious mononucleosis" has been used by Sprunt and Evans to describe this condition. Bloedorn and Houghton have used the term "acute benign lymphoblastosis," and Downey has called this disease "acute lymphadenosis." The abnormal cells encountered in stained smears more closely resemble the germinal center type of lymphoblasts and the Rieder type cell than the large mononuclear cell normally found in the blood. These lymphoblasts are often mistaken for mononuclears and myeloblasts, but differ in that they do not show a peroxidase ferment, as demonstrated by Goodpasture's stain. Longcope and others have noted the frequent association of organisms of Vincent's angina in the throats of patients with this condition. It is important to distinguish this disease from acute lymphatic leukaemia, as these patients usually recover in the course of a few weeks.

Hodgkin's Disease.—In this disease the excision of one of the enlarged lymphatic glands gives us our best method of diagnosis. In the very early stages there is mainly an increase in the normal lymphocytes with some degree of endothelial cell proliferation. Later on this increases and we begin to have peculiar multinucleated cells which resemble tuberculous giant cells, but showing fewer and larger nuclei. About this time a fibrosis is noted which tends to increase until it makes up the greater part of the pathology. Eosinophiles may be quite abundant. The spleen usually enlarges. Hodgkin's disease has an insidious onset, the enlargement of the gland on one side of the neck usally first attracting attention. The axillary glands of the side showing neck involvement are apt to show enlargement. Later on we may have an encircling collar of neck glands. Rarely, the first glands to enlarge may be the internal ones. Some cases show a relapsing type of fever. The red cells slowly decrease as the disease progresses, with corresponding or slightly greater haemoglobin decrease, so that the color index tends to be below 1. There is usually a moderate white cell increase (10,000–15,000) with 7 to 10% of transitionals and an occasional myelocyte. Blood platelets are increased.

Kundrat's Lymphosarcoma.—In this affection the glandular involvement begins locally but develops more rapidly than in Hodgkin's disease. It is much like lymphosarcoma in that it extends to adjacent glands but does not tend to invade neighboring organs. The spleen and liver are rarely involved. The blood shows an anaemia with a neutrophilic leukocytosis and diminution of lymphocytes.

Lymphoid Pseudoleukaemia.—It is difficult to differentiate this condition from true lymphatic eukaemia when aleukaemic. The white count varies from 5000 to 10,000 with about 75% of lymphocytes. The spleen is enlarged as well as the lymph nodes and there is a tendency to haemorrhage in the later stages.

Multiple Myeloma.—In this disease we have more or less generalized tumor formation in bones, especially the ribs and vertebrae. The tumor cells seem to be derived from normal marrow cells and differ in different cases. The finding of Bence-Jones body in the urine is important diagnostically. The disease starts in with neuralgic pains and weakness. The blood shows very little that is of importance but at times there may be a great increase of Türck irritation forms. The X-ray is of value in bringing out the bone lesions.

Chloroma.—This is a disease characterized by green-colored nodules about the cranial bones. These subperiosteal nodules may be present in cases of the leukaemias.

Granulomatoses.—These cases are generally of tuberculous or luetic origin. They do not show the tendency to haemorrhage of lymphoid pseudoleukaemia. They may show a neutrophile leukocytosis.

The Splenomegalies

The only diseases associated with splenic enlargement to be considered under this heading will be those to which the term "splenic anaemia" has been loosely applied, viz: Banti's disease, Gaucher's splenomegaly, splenic anaemia of infants and the two types of haemolytic jaundice. In the differential diagnosis of diseases associated with splenomegaly it is well to have in mind the many conditions in which it occurs besides those previously considered and those noted immediately above. The splenic enlargements connected with various acute infectious diseases rarely cause confusion but the chronic splenomegalies of tuberculosis, syphilis and portal obstruction must be thought of when a diagnosis of leukaemia, pseudoleukaemia or splenic anaemia is being considered. Prolonged suppuration may give rise to an amyloid spleen. Of tropical diseases, the best known splenomegaly is "ague cake" of chronic malaria. In first attacks of malaria the splenic enlargement is not much greater than in typhoid fever. Both the adult and infantile types of leishmaniasis give a much enlarged spleen. Undulant fever and relapsing fever are also associated with more or less splenomegaly.

Banti's Disease.—We do not know the cause of this disease but it seems to originate from disordered function of the spleen, which may be of the nature of increased haemolysis, or from depression of bone marrow activity. The spleen shows a fibrosis with atrophy of the Malpighian corpuscles. The disease usually starts in early adult life and for several years there may only be splenomegaly, anaemia, weakness and alimentary tract disturbances. Along with a developing cirrhosis of the liver, increase in the digestive disturbances and progressive emaciation and anaemia mark the third stage in which the chief clinical manifestations are those of cirrhosis of the liver with ascites. Very brilliant results have been obtained from

splenectomy even when the disease has lasted for a long period. The red cell count is not lowered as much as would be expected and counts of about 3,000,000 may be found even when the anaemia and weakness are marked. There is a tendency to haemorrhages. At first the leukocytes are unaffected; but later on the polynuclears diminish in percentage and, in the later stages, there is a well marked leukopenia involving lymphocytes as well as polynuclears. In some cases there is a considerable increase of the large mononuclears, but myelocytes and nucleated reds are very rarely encountered in a blood film. On the whole, with the exception of a certain degree of leukopenia, the blood findings are negative.

Gaucher's Disease.—This, often referred to as the Gaucher type of Banti's disease, is very rare. The disease begins in childhood and runs a chronic course, covering many years. The splenic enlargement is often stated to be due to an endothelial cell proliferation but most authorities regard the characteristic cell increase as of origin other than endothelial. The peculiar cells, large vesicular ones with a small eccentric nucleus, fill the venous sinuses. Similar cell aggregations are found in the liver, bone marrow and lymphatic glands. The anaemia, like that of Banti's disease, is not extreme. A brownish or greyish skin discoloration and thickening of the conjunctivae may be present. There is usually a leukopenia with a tendency to the appearance of normoblasts and myelocytes in the blood film.

Splenic Anaemia of Infants.—This, v. Jaksch's disease, is a splenomegaly of children, usually under two years of age. Some cases are probably connected with congenital syphilis and some with infantile leishmaniasis (Italy). There is a very high grade anaemia with some of the findings of pernicious anaemia. Many cases show a polynuclear leukocytosis and some a rather marked lymphocytosis.

Haemolytic Jaundice.—Two types are recognized, one of the family form and the other an acquired type. In the acquired type the onset is more stormy and the anaemia more severe. It is stated that the acquired form shows autoagglutination which is negative for the congenital type. Both types have an acholuric jaundice which may show periodic increase. The anaemia approximates two million red cells for the acquired and three million for the congenital. The most marked blood feature is the diminished resistance of the red cells to hypotonic salt solution (0.64 to 0.62% NaCl) and the presence of reticulated red cells.

Icterus is present in haemolytic jaundice, but rare in other forms. The resistance of the red blood cells is normal in Banti's disease, increased in pernicious anaemia and diminished in haemolytic jaundice. Reticulated red cells are normal in Banti's disease and increased in haemolytic jaundice and pernicious anaemia. The liver is usually enlarged in all the above diseases, except pernicious anaemia, but may be normal in acquired haemolytic jaundice, and is contracted in the later stages of Banti's disease.

THE SPLEEN AND ANAEMIA. DIFFERENTIAL DIAGNOSIS. (PEARCE, KRUMBHAAR AND FRAZIER)

	Pathology of spleen	Time of onset	Duration	Splenomegaly	Haemorrhages	Anaemia
Gaucher's disease	Peculiar cell hyperplasia	Childhood	Many years	+++	Occasionally	Slight
Banti's disease	Hyperplasia fibrosis	Adult life	Few years	+	Occasionally	Slight to severe
v. Jaksch's disease	Hyperplasia fibrosis	Infancy	Several months	++	?	Slight to severe
Haemolytic jaundice (acquired)	Congestion and pigmentation	Any age	Many years	++	Rare	Severe
Haemolytic jaundice (congenital)	Congestion and pigmentation	Congenital or childhood	Many years	++	o	Slight
Pernicious anaemia	Fibrosis	Adult life	Few years with remissions	Slight or diminished	Rare	Severe

PART III

ANIMAL PARASITOLOGY

CHAPTER XV

GENERAL CONSIDERATIONS OF CLASSIFICATION AND METHODS

Animals that are in all respects alike we term a species. Of course the male and female of a species may be very unlike, but as a result of mating they produce young having characteristics similar to the parents. Now if, as in the case of the mosquitoes, we find some with straight silvery lines and others uniformly showing crescentic silvery bands about thorax, yet resembling each other closely in the respect of being small, dark, brilliantly marked mosquitoes, we should consider them as being separate *species* with a certain relationship to which the term *genus* is applied.

The term "genus" is of wider application than the word "species." Thus animals which agree in the main characteristics of size, proportion of parts, and general structure are placed in the same genus.

In naming a species we always first write the name of the genus which has a Greek or Latin name, commencing with a capital, and follow with the specific name, which latter commences with a small letter. Thus we designate the common tropical house mosquito that is active at night, *Culex quinquefasciatus*. This species is characterized by the failure of the abdominal bands to reach the sides, especially posteriorly, thus differentiating it from a closely related species in colder climates, *Culex pipiens*, in which species the abdominal bands reach the sides.

For the generic name to be valid it must be one that has not already been given to another group of animals. The definition of a genus is based on the first described species of the genus, which is designated the type species or genotype, and if at any time such a genus is broken up into other genera this genotype remains with the original genus.

When a valid name has been given a species, and that species is later on transferred to another genus, the specific cognomen must attach to the new genus; thus *Culex aegypti* when placed in the genus *Stegomyia* becomes *S. aegypti* and when placed in the genus *Aedes* becomes *A. aegypti.*

The specific name may be a noun in the genitive. If an adjective it must agree in gender with the generic name.

It is permissible to have a masculine noun as a specific name with a feminine generic name.

If the specific name is a modern patronymic we add *i* in the case of a man or *ae* for a woman to the exact and complete name of the person.

Again certain genera show resemblances which enable us to make broader groupings to which we apply the term tribe. Thus the genera *Aedes* and *Culex* have certain similar larval characteristics and the imagos a nude metanotum; we therefore classify all species of these genera under the designation *Culicini.* The name of a tribe ends in "*ini.*" Now, again, certain insects are different from others in having a long proboscis adapted for piercing. We find that not only do the *Culicini* have such characteristics but the same is observed with the *Sabethini.* These we term a subfamily and we speak of the *Culicinae,* meaning the true mosquitoes. The name of a subfamily ends in "*inae.*" Again, certain insects differ from others in having scales on their two wings. Thus we find that not only do the *Culicinae* (true mosquitoes) have such characteristics but also the *Corethrinae.* These together we term a family and speak of them as the *Culicidae.* The name of a family ends in "*idae,*" this suffix being added to the inflexional stem of the name of the first named genus of the family. Many families are not subdivided into subfamilies but are directly separated into genera. Again a genus may have only a single species.

At times a family may be raised to *superfamily* rank—the subfamilies then becoming families. Thus the families Ixodidae and Argasidae belong to the superfamily Ixodoidea. The termination for a superfamily is "*oidea.*"

When there are a number of families agreeing closely in some striking characteristic, we group them together into an *order;* thus, the family of mosquitoes closely resembling many other families of insects in possessing a pair of well developed wings is grouped in the order Diptera, all of which resemble certain other animals in the possession of a distinct head, thorax and abdomen with three pairs of legs projecting from the thorax. This collection of animals we call a *class;* thus, we speak of the class Insecta. It will be observed that the insects have no internal skeleton, but instead a chitinous cuticle, the exoskeleton. Spiders, ticks, etc., resemble them in this respect, and we now apply to all such animals the wider designation, *branch* or *phylum* Arthropoda.

Inasmuch as the animal kingdom is divided into the branches Protozoa, Porifera, Cnidaria, Echinodermata, Vermes, Annelida, Arthropoda, Mollusca and Chordata, we see that the branch is the largest grouping we employ. To descend in the

scale we have belonging to the branch, the classes; to the class, the orders; to the order, the families; to the family, the subfamilies; to the subfamily, the tribes; to the tribe, the genera; to the genus, the species. Occasionally a species is further divided into subspecies.

By a *type species* we understand the species of a genus always referred to as representing the genus.

Many favor alliteration for type species, as *Heterophyes heterophyes*.

The male animal is designated by the sign of Mars (♂), the female by that of Venus (♀).

Classification of Animal Parasites

(According to Stiles)

1. Unicellular animals (without tissues), as the parasites of malaria.....Protozoa
   Pluricellular animals (with tissues); metazoa..... 2
2. Body more or less flattened dorso-ventrally..... 4
   Body ordinarily round in transverse section..... 3
3. Body never annulated; never provided with legs; jaws absent..... 5
   Body annulated, or at least provided with mouth parts; usually breathe through a tracheal system; adults with jointed legs or other appendages..... 7
4. Intestine, but no anus, present; one or two suckers present; body not segmented; parasitic in liver, lungs, blood, intestine, occasionally elsewhere; flukes..... Trematoda
   Intestine absent; two or four suckers on head; body of adults segmented; tissue usually contains calcareous corpuscles; adults (tapeworms) parasitic in intestine; larvae (bladder worms) parasitic elsewhere.....Cestoda
   Intestine and anus present; sucker on posterior end; body annulated like an earthworm; parasitic in upper air passages, or externally; leeches, blood suckers... Hirudinea
5. Intestine absent; armed rostellum present; very rare in man, in intestine; thorn headed worms.....Ancanthocephala
   Intestine present; no armed rostellum.....Nematoda 6
6. Intestine rudimentary in adults; lateral chords absent; rare, accidental parasites in intestine of man; hair snakes or horse-hair worms.....Gordiacea
   Intestine present; lateral chords present; parasitic in intestine, muscles, lymphatics, etc., very common and important; roundworms.....Eunematoda
7. Six legs present in adult; wings present in most species; larva annulated much like an earthworm; breathe through trachea; adults ectoparasites; occasionally larva is parasitic under skin, or in wounds, or an accidental parasite in the intestine or bladder; insects.....Insecta
   Eight legs present in adult, six legs in larva; head and abdomen coalesced; ectoparasites; some burrow under the skin or live in the hair follicles; acarines.... Acarina
   Four claws around the mouth; larva encysted in various organs; adult occasionally parasitic in nasal passages; tongue worms.....Linguatulidae
   Numerous legs present; occasionally accidental parasites in nasal passages or intestine; thousand leggers.....Myriapoda

General Considerations of Animal Parasitism

While it is usually comparatively easy to determine whether a parasite be vegetable (bacteria, moulds) or animal, yet there are many parasites about which a doubt exists as to their true position. This is particularly true of the group of spirochaetal organisms causing such important diseases as syphilis, yaws, relapsing fevers, yellow fever and infectious jaundice.

As noted in the preceding table of phyla the animal parasites of man belong to protozoal, helminthological and arthropod branches. Among protozoal and arthropod animals the free-living forms make up a large proportion of such branches, but with the helminthes the state of parasitism is almost universal. An exception to this is the class Turbellaria (planarians) which, in contrast with the cestodes, trematodes and nematodes, are free-living, usually found swimming about in salt or fresh water or occasionally found in moist earth.

The more adapted to parasitism an animal becomes the more striking the change in structure from that characterizing free-living forms. In particular does the locomotor apparatus degenerate, and frequently it is replaced by fixation organs, such as suckers or hooklets. At the same time the muscular and nervous systems undergo degeneration, and with the entozoal parasites the furnishing, by the body fluids or tissues of the host, of suitable food for the parasite brings about simplification or disappearance of the digestive apparatus. So great may be the degeneration of structural parts that certain parasites, as the tongue-worms (Linguatulida), which are arachnoids, resemble tape-worms more than they do the mites and ticks to which they have a classificatory relation.

When we consider the reproductive system of obligate parasites we find extraordinary development. It will be evident that should the entire life cycle of an animal parasite be passed in a single animal the continuation of the species would be endangered—the parasites would die when the host ceased to live.

For this reason the transfer of the parasite to another individual is necessary, or a substitution of hosts, or adoption of free-living periods. The chances of finding suitable intermediary hosts and of a return to the original one must be very slight for an individual and to provide against possibilities of extinction there is prodigality in reproduction. In the case of the lung fluke and certain other flukes three hosts are connected with the life history of the parasite. Such animals as flukes and tape-worms seem to be made up largely of sexual organs and accumulations of eggs. The mature segments of *Hymenolepis nana* show solely a mass of eggs.

Parasites damage the human host in various ways. Some consume the food supply in the alimentary tract to such an extent that malnutrition may result. Others actually destroy important tissues or organs. There may be such a loss of blood by the wounding of blood vessels incident to the wanderings of the parasite or in satisfaction of food demands that marked anaemia results.

Some parasites elaborate toxic material. There is also the probability that the traumatism to intestinal or other structures may provide an atrium for bacterial infection.

At times we divide parasites into ectoparasites and endoparasites, according as they live upon or within the body of the host. It would be an easy matter to decide that the body louse belonged to the first group and the hookworm to the latter, but with the itch mite, which penetrates the skin, or the chigoe, which burrows under the toe nail, it would be a matter of difficulty.

It is usual to use the term infection for parasitization within the body, as a flagellate infection, and infestation for that on the surface of the body, as an infestation with body lice. There are certain authorities who would call helminthic diseases infestations but this use of the term is not common.

While the life history of those parasites which show a direct transmission, as from man to man, or reinfection of the same individual, is on the whole simpler than that of those requiring one or more intermediate hosts, yet, even with the former there is great variation in complexity of life cycle.

Take the case of *Enterobius* (*Oxyuris*), the pin-worm, where the mature female leaves the intestines to crawl upon perineal surfaces. The uterus contains embryo-filled eggs, which contaminate the fingers when the parent worm is disintegrated as a result of scratching the parts visited by the worm. These eggs when introduced into the mouth are ready to develop into adults in the intestine. In the case of *Ascaris*, however, the eggs must gain access to moist soil for 5 or 6 weeks, until an embryo has developed within the shell, when they prove infective upon ingestion. With the hookworm egg not only must an embryo develop within the egg shell like that for *Ascaris* but the embryo must leave the shell and undergo moulting and changes in form before it is capable of boring its way into the skin later to become mature in the intestinal tract. With those parasites requiring one or more intermediate hosts the problem is still more complex. The cycle may vary from the relatively simple case of *Taenia solium* in which the embryo develops in the muscles of the hog and the adult tape-worm in man to the more involved cycle of *Paragonimus ringeri* in which the embryonal stages are passed in the snail and then in the crab and the adult develops in the lungs of man.

When intermediate hosts are essential to the survival of a parasite, their geographical distribution becomes an important factor in epidemiology. Man with the infection may be present in a locality, but without the coexistence in the same locality of the intermediate host the parasite cannot be transmitted. Cases of sleeping sickness were introduced into the new world from Africa with the slaves but as the tsetse fly was not present in the new world there was no transmission. With ancylostomiasis an intermediate host was not required and we had the spread of this infection to North and South America.

The generally accepted view that some races show less susceptibility to certain parasitic infections has been questioned. An examination of the stools of 26,672 white soldiers and 8653 colored ones coming almost exclusively from

Kentucky, showed a ten times greater incidence of intestinal parasites in the whites than in the blacks. The ratio for *Necator* was 12 to 1, for *Ascaris* 7.8 to 1, for *Trichuris* 16.6 to 1 and for *Hymenolepis* 3.5 to 1. This difference of parasitic infection would be difficult to explain on the basis of more hygienic living on the part of the blacks, although it may be stated that the more hygienic living of Europeans is generally given as the reason for the greater incidence of parasitic diseases in China or the Pacific Islands than in Europe.

There are certain terms employed in animal parasitology which it is necessary to understand. Among these we shall refer to the following:

1. **True Parasitism.**—By this is understood the condition where the parasite does harm to the host, deriving all the benefit of the association. A good example of this would be the hookworm infecting man or animals.

2. **Mutualism.**—In such an association there is mutual benefit to each party of the association. An instance of this would be the presence of colon bacilli in the intestines. The bacillus is furnished a suitable habitat and in return protects its host against strictly pathogenic bacteria.

Another example would be the oyster crab found inside the oyster shell.

3. **Commensalism.**—Here there is benefit to the parasite, but no injury to the host. An example of this kind would be furnished in the case of the *Trichomonas vaginalis* which lives in the vaginal mucus, but so far as known, does no injury to the host. If the *Endamoeba coli* be nonpathogenic this would be another example.

4. **Hyperparasitism.**—In human parasitology we concern ourselves with primary parasites, but in economic entomology the existence of parasites parasitizing the injurious parasite is well recognized.

The secondary parasites of the cotton worm practically annihilated the fifth brood of this pest. Tertiary parasites are also recognized but these, by destroying the beneficial secondary parasites, would favor the original parasite.

This is a field of human parasitism which might well be given more consideration, especially with parasites which do not respond to chemotherapy.

5. **Nomenclature.**—When the thousands of different species, genera, etc., of animals are considered, it will be readily perceived that, unless some system existed for their designation, indescribable confusion would prevail. To avoid this, the International Code, based on the rules of Linnaeus (tenth edition of Systema Naturae, 1758), is made the basis of binary zoological nomenclature.

In printed matter the zoological name should be in italics, that of the family in Roman type. Names should be in Latin, or be latinized. The name of the author of a specific name is written immediately after the name without punctuation and may be followed by the year of publication set off by a comma, thus; *Ascaris lumbricoides* Linnaeus, 1758. Should the name of the author appear in parentheses it indicates that he proposed the specific name but placed the species in another genus than that in which it now appears, and the name of the author responsible for placing the species in the present genus may be written after the name of the original author of the species; for example, "*Davainea madagascariensis* (Davaine, 1869)

Blanchard, 1891," tells us that Davaine proposed the specific name *madagascariensis* in 1869 but placed it in some other genus and that Blanchard in 1891 transferred it to the genus *Davainea*. There are certain rules governing the naming of animals. Of these, the law of priority provides that the oldest published name, under the code, of any genus or species is its proper zoological name. The name must appear in a well recognized publication and even the printer's proof does not establish priority. The history of the naming of the organism of syphilis illustrates this well.

The law of priority and the name of the cause of syphilis.—Schaudinn gave this organism in 1905 the name of *Spirochaeta pallida*. Ehrenburg, in 1834, had used the name *Spirochaeta* for protista of a different character, so that this designation of the genus was not permissible under the code. Later on in 1905 Vuillemin proposed the generic name *Spironema*. This name, however, was found to have been used in 1864 by Meek for a genus of mollusks and by Klebs in 1892 for a genus of flagellates. Consequently, being a homonym, it was not available.

(A generic name can be applied to only one animal genus and if a similar name is subsequently given another genus it is a homonym and is to be rejected.)

On Dec. 2, 1905, Stiles and Pfender then proposed the name *Microspironema* but as Schaudinn published on Oct. 26, 1905, the designation *Treponema*, the name *Treponema pallidum* had to be accepted as the proper zoological name for the organism of syphilis.

The question of the name of the organism of relapsing fever.—The generic name *Spirochaeta* not being available, Noguchi, in 1918, proposed the name of *Spironema*. As noted under the discussion of the naming of the organism of syphilis this name was not valid. In November, 1907, Sambon proposed the name *Spiroschaudinnia* for the blood spirochaetes and this name would seem the proper one were it not for the fact that in July, 1907, Swellengrebel proposed the name *Borrelia* for the group of organisms of which *B. gallinarum* was the genotype. Sambon's classification in which he used *Spiroschaudinnia* for blood spirochaetes was presented in a paper read before the British Medical Association in July, 1907, but as it was not published until November, 1907, this is the time of priority. It is the publication in an acceptable journal and not the time a paper is read that gives basis for priority. Swellengrebel differentiated *Borrelia* from *Treponema* on the basis of peritrichal flagella for the spirochaetes of the blood—a conception which has not been confirmed. This, however, does not invalidate the name because the International Commission holds that we name the objects themselves not our conception of said objects and Swellengrebel named spirochaetes of which *gallinarum* was the genotype as *Borrelia*.

Even if *Borrelia* could be rejected on the ground of an erroneous definition the same would hold true for *Spiroschaudinnia* which Sambon stated to form sporozoites—also an error in conception.

The name of the mosquito transmitting yellow fever.—The name of the yellow fever mosquito, so long established as *Stegomyia fasciata*, has as a consequence of the operation of the law of priority been changed several times during the last few years, older names designating the species having been found in the literature. The generic name *Culex* which in former times was used for the entire mosquito family was replaced in 1901 (Theobald) by the generic name *Stegomyia*. Recent authorities state however that the generic characteristics of *Stegomyia* are not distinctive enough to warrant the formation of a separate genus, and for this reason the generic

name *Stegomyia* was dropped and replaced by *Aedes*, a reconstructed genus that included amongst others the various species of *Stegomyia*. At present, however, there is shown a marked tendency to subdivide the genus *Aedes* into several subgenera one of which is *Stegomyia*, so that the proper generic name for the yellow fever mosquito then would be *Aedes* (*Stegomyia*).

The specific name of this mosquito presents a more varied history. In all works on mosquitoes a large number of synonyms are given for this species, some of which are, *albopalposis*, *fasciata*, *persistans*, *nigeria*, *elegans*, *bancrofti*, *excitans*, *etc.*; but these various names have all been dropped because the species had been described and named prior to their introduction. Up to a few years ago discussions of this mosquito considered only two names, namely *fasciata* and *calopus*, Fabricius having named the species, *Culex fasciatus* in 1805 and Meigen *C. calopus* in 1818. Priority indicated that *fasciatus* should be the specific name but according to some authorities this name was preoccupied (Müller, 1764), thus making *calopus* the valid name. Further investigation has shown however that the claims for preoccupations of *fasciata* were not well founded, and hence this name would be the proper one, if it were not for some recent findings which show that Poiret named the species, *C. argenteus* in 1787 and Linnaeus, *C. aegypti* in 1762. The descriptions of the mosquito given by these men undoubtedly indicate that they were dealing with the species in question and hence according to the law of priority the name given by Linnaeus is the proper one.

Thus the name of the yellow fever mosquito as established today is *Aedes* (*Stegomyia*) *aegypti* Linnaeus.

Of unusual interest is the question of the name of the old-world hookworm. Dubini, in 1843, named a nematode found by him in man *Agchylostoma*. By the law of priority this spelling would have been the correct one had he not stated in a footnote that the generic name was derived from two Greek words *αγχὺλοσ* and *στὸμα*. Having indicated the origin of the name it became subject to the rules for correct transliteration, which is *Ancylostoma*.

In case of larva and adult, or male and female, formerly considered different animals but subsequently found to be the same, the oldest available name becomes the name of the species.

Another point is that names are not definitions, consequently the fact of lack of appropriateness of any name is no objection to its continuation. This will appeal to anyone as a wise provision, because if a different name were substituted each time a designation more descriptive or applicable was invented it would be utterly destructive to system. When it is considered that some of our parasites have approximately fifty different designations, for the most part given by medical observers, it will be appreciated how much the zoologist has aided us in trying to eliminate all but the single proper zoological name.

It is a rule of zoological nomenclature that zoological names are independent of botanical ones so that the prior use of a generic name for a plant is not a valid objection to its use for an animal, but it is well

to avoid introducing into zoology as generic names such as are already in use in botany.

The objections so frequently heard among physicians in connection with adopting new names for old ones are not well founded. Wherever confusion has reigned, the establishment of order always results in temporary greater confusion. There is no doubt that the student taking up this subject a few years hence will have the satisfaction, thanks to the zoologist, of having to burden his mind with only one name for each parasite.

There is only one correct name for an animal and all other names are synonyms.

The principal cause of changes of names is that our conception of the relationships of animals changes.

6. **Terminology.**—This applies to appropriate designations for different organs, symptoms, etc., and is not subject to any rule other than that of good usage.

Thus the terms cirrus in the case of male copulatory organ of flukes, spicule for the same in nematodes and penis in connection with insects would be instances of terminology.

7. **Pseudoparasitism.**—Where organisms enter the body accidentally and when such sojourn in the body of man plays no part in the life history of the organism we employ the term pseudoparasitism. For example: Fly larvae swallowed by man and passed out in the faeces. We also use the terms temporary parasites (bedbug) and permanent parasites (liver fluke).

8. **Hosts.**—The animal in which a parasite undergoes its sexual life is called the definitive or final host, that in which it passes its larval existence the intermediary host. For example: Man is the intermediary host of the malarial parasite, the mosquito the definitive host. A single animal may, however, be both definitive and intermediary host; thus, *Trichinella* may pass its larval existence in the muscles of man and its sexual life in his intestines. With certain infections we have two intermediate hosts as in paragonimiasis where the first intermediate host is a mollusk and the second intermediate one a crab.

9. **Metaxeny.**—This is a term recently introduced to express the conditioned existence of a parasite upon an intermediate host. Examples are furnished in the dependence of malarial parasites on the human host and of the schistosomata on mollusks.

10. **Heredity, Congenitalism.**—Hereditary characteristics are those which were present potentially in the ovum or spermatozoon before fertilization; congenital ones those which originate after fertilization but before birth. South African tick fever is probably an instance of heredity, the spirochaetes having been found in the ovary and ova of the female tick.

11. **Heterogenesis, Parthenogenesis.**—Offspring differs from parent, but after one or more generations there is reversion to the parent form.

Strictly speaking, the term heterogony applies to reproduction when a sexual generation alternates with a parthenogenetic one. Where a nonsexual generation, as by division or budding, alternates with a sexual one, the process is called meta-

genesis. In parthenogenetic reproduction, eggs develop without fertilization by spermatozoa.

In coccidiosis we have a sexual cycle (sporogony) alternating with a nonsexual one (schizogony). In the infection with *Strongyloides* we have a sexual cycle alternating with a parthenogenetic one. In malaria we have a sexual generation, a nonsexual one and, according to Schaudinn, a parthenogenetic one.

12. **Homology and Analogy.**—By homology we understand the anatomical correspondence of the organ of one animal to that of another. Thus the fore leg of a quadruped and the wing of a bird are homologous organs. Analogy refers to physiological or functional agreement; thus the lungs of mammals and gills of fish, both with respiratory functions, are analogous organs. The first trace or appearance of an organ in an embryo is known as the *anlage of the organ.*

13. **Protista.**—Haeckel proposed this name for unicellular animals and plants, thus including protozoans and protophytes in a kingdom separate from the animal and vegetable kingdoms.

We have sufficient difficulty in drawing the line between an animal and vegetable organism and to make a demarcation of a new kingdom from the two usually recognized would add to our difficulties.

14. **Phylogeny and Ontogeny.**—Phylogeny deals with the evolution of a group of animals. The phylogenetic or ancestral history of the genesis of the horse of the present day shows that it developed from an animal with four toes on the fore foot and three on the hind foot. Ontogeny deals with the evolution or germ history of an individual. It deals with the development from egg to mature adult.

Epidemiological Considerations

Some apply the term *intermediary* host to one in which the parasite does not undergo development and restrict *intermediate host* to one in which a nonsexual cycle takes place. It is probable that the definitive host is the original host of the parasite and as a rule such a host does not directly suffer from the harboring of the parasite. When a parasite leaves its definitive host it frequently assumes a resistant stage and as such may be taken up by another host and continue as such in this second host, without multiplying or producing ill effects in such second host. This parasite, which is simply protected by the host, and does a host no damage, is an example of commensalism. This is exemplified in the carriage of endamoebic cysts by the house fly. The cysts leave the human alimentary tract as such and after a period of residence in the house fly leave this protective host, in its faeces, to be subsequently ingested by man and resume the developmental life which causes dysentery. With the parasites which undergo a nonsexual multiplication in the second host we have an instance of a true intermediate host and as a rule such a host is damaged by the parasite. For instance, *Plasmodium vivax* produces disease in its nonsexual life in man, its intermediate host, but apparently does no harm to its definitive host, the anopheline mosquito, while undergoing its sexual cycle. In the same way *Wuchereria* (*Filaria*) *bancrofti* does not directly harm man, its definitive host, but when undergoing its nonsexual life in the culicine mosquito it is inimical to the life of the mosquito. While, as a rule, no harm is done man by the filarial infection and, as is well known, the peripheral blood of a human host may teem with

filarial embryos without his showing any ill effects, yet with secondary factors (pyogenic organisms producing lymphangitis or effects of lymphatic obstruction) we may have clinical manifestations of disease.

Transmission of Parasites.—Where the definitive and intermediate hosts are both vertebrates, the passage from one to the other is ingestive; thus cattle take in the onchosphere of *Taenia saginata* at the time of grazing and man subsequently (eats the raw or insufficiently cooked meat of such cattle containing the embryonic parasite (*Cysticercus bovis*). When transmission is from a vertebrate to an arthropod the latter take the parasite into its alimentary tract so that the process is ingestive. With the transfer of a parasite from arthropod to vertebrate this may be ingestive, as with endamoebic cysts from the fly, but is more often inoculative, as when man is bitten by a transmitting tick, mosquito or fly. When there is no change in the parasite, but simply a direct transfer from the biting parts of the fly, recently contaminated by feeding on one vertebrate, to another vertebrate we have a *direct inoculative transmission*. This is recognized for certain other animals but probably does not occur in the case of man. When the parasite undergoes a developmental or cyclical change in the arthropod and is subsequently injected into man by the biting process we have an *indirect inoculative method of transmission*.

Reservoir of Virus.—This is a very convenient term to employ in discussing epidemiology. By this is understood the source from which a host becomes parasitized. A man having sexual malarial parasites in his blood is a reservoir of virus for the parasitization of anopheline mosquitoes which may take up his blood. In malaria, man is the only reservoir of virus, but in some diseases some other animal as well as man may be a reservoir of virus. It is considered that wild antelope may be the reservoir of virus for *Trypanosoma rhodesiense* as well as man. These reservoirs may provide material for the parasitization of either the definitive or the intermediate host.

CHAPTER XVI

TABLE OF IMPORTANT ANIMAL PARASITE DISEASES

Protozoal Diseases

Parasite	Defin. host	Intermediate host	Important reservoir of virus	Transmission and pathogenicity
Endamoeba histolytica.	Man.	Not required.	Man-carrier stage (faeces).	Cysts in food or water. Flies may act as carriers. Ingestive. Amoebic dysentery.
Balantidium coli.	Man (hogs).	Not required.	Man-carrier stage (hogs).	Transmission probably same as for E. histolytica. Found in those having care of hogs. Ingestive. Balantidium dysentery. Anaemia.
Giardia lamblia (Lamblia intestinalis).	Man (mice and rats).	Not required.	Man-carrier stage (mice and rats).	Transmission probably same as for E. histolytica. Rat faeces on human food important. Ingestive. Lamblia dysentery. Giardiasis.
Borrelia recurrentis, carteri, etc. (Spiroschaudinnia recurrentis, carteri, etc.) (Louse group.)	Louse (P. vesti-menti).	Man.*	Man (blood).	Cyclical development in louse. Bite puncture contaminated by crushed louse. Relapsing fever.
Borrelia duttoni, novyi. (Spiroschaudinnia duttoni, novyi.) (Tick group.)	Tick (species of Ornithodorus or Argas).	Man.*	Man (blood).	Excretions of tick contaminating tick-bite, Tick fevers. Relapsing fever.
Treponema pallidum and pertenue.	Man.	Not required.	Man.	T. pallidum. Usually venereal. Syphilis. T. pertenue. Flies or contact. Yaws.
Leptospira icterohaemorrhagiae.	Man (rat).	Not required.	Rat.	Common infection of rats. Present in blood. Excreted in urine. Ingestion. Weil's disease.

PROTOZOAL DISEASES (Continued)

Parasite	Defin. host	Intermediate host	Important reservoir of virus	Transmission and pathogenicity
Leptospira icteroides.	Mosquito Aedes aegypti (Stegomyia fasciata.)	Man.	Man (blood).	Cyclical development in mosquito—12 days. Inoculative. Yellow fever.
Leptospira morsus-muris.	Man (rat).	Not required.	Rat.	Man inoculated by bite of infected rat. Rat bite fever.
Trypanosoma gambiense and rhodesiense.	Fly (Glossina species).	Man.	Man—game animals? (blood).	Cyclical development in tsetse fly. Inoculative. Sleeping sickness.
Schizotrypanum cruzi.	Lamus megistus.	Man.	Man.	Cyclical development in bug. Inoculative. Brazilian trypanosomiasis.
Leishmania donovani, infantum, tropica and braziliensis.	Not surely known.	Man.	Man.	L. donovani—species of Phlebotomus? kala-azar. L. infantum—dog flea? infantile leishmaniasis. L. tropica—species of Phlebotomus? Oriental sore. L. braziliensis—biting insects? American leishmaniasis.
Plasmodium malariae, vivax and falciparum.	Mosquito (Anopheline species).	Man (with schizonts).	Man (blood) (with gametocytes).	Cyclical development in mosquito—12 days. Inoculative. Malaria.
Rickettsia prowazeki.	Man.	Louse (P. vestimenti).	Man (blood).	Cyclical development in louse. Bite puncture inoculated by louse faeces. Typhus fever.
Dermacentroxenus rickettsi.	Man (goats, rodents, etc.).	Tick (Dermacentor andersoni).	Goats and rodents.	Excretions of tick contaminating tick bite, Rocky Mountain fever.
Bartonella bacilliformis.	Man.	Unknown.	Man (blood).	Transmission unknown. Species of Phlebotomus suggested. Carrion's disease.

NOTE.—*Some authorities give man as definite host of Borrelia (Spiroschaudinnia). Diseases often classified as filterable virus ones of protozoal affinity are: (1) Dengue. Cause unknown. Transmitted by Culex (?) and Aedes species. (2) Pappataci fever. Cause unknown. Transmitted by Phlebotomus species. Other protozoal diseases are: (1) Tsutsugamushi. Probably a protozoan. Transmitted by Kedani mite. (2) Trench fever. Probably Rickettsia. Transmitted by louse. Noguchi considers B. bacilliformis a bacterium.

HELMINTHIC DISEASES—TREMATODES

Parasite	Defin. host	Intermediate host	Important reservoir of virus	Transmission and pathogenicity
Clonorchis sinensis.	Man (cats, dogs and hogs).	1st, snail (species of Melania and Bythinia) and 2d, fish.	Man.	Eating raw fish. Ingestive. Human liver fluke disease.
Opisthorchis felineus.	Man (cats and dogs).	1st, mollusk. (Dreissena polymorpha?) 2d, fish.	Cats and dogs.	Man probably infected by eating raw fish. Ingestive. Liver fluke disease.
Fasciolopsis buski.	Man (pig).	Species of Hippeutis and Segmentina.	Hog.	Ingestion of cercariae encysted on water plants. Intestinal Distomiasis.
Heterophyes heterophyes.	Man (dogs and cats).	1st, probably mollusk, 2d, probably fish.	Dogs and cats.	Not definitely known. Intestinal Distomiasis.
Heterophyes nocens.	Man and probably other animals.	Fish.	Man (faeces).	Eating raw fish containing the encysted larvae.
Paragonimus ringeri.	Man (dogs, cats and hogs).	1st, snail (species of Melania). 2d, crab.	Cats, dogs and hogs.	Eating raw crabs containing cercariae. Ingestive. Lung fluke disease.
Schistosoma haematobium.	Man.	Snail (species of Bulinus (Isidora?) and possibly others).	Man (urine).	Bathing or drinking water containing cercariae. Penetrative. Vesical bilharziasis.
Schistosoma mansoni.	Man.	Snail (species of Planorbis) and possibly others.	Man (faeces).	Bathing or drinking water containing cercariae. Penetrative. Rectal bilharziasis.
Schistosoma japonicum.	Man.	Snail (species of Katayama and Oncomelania).	Man (faeces) (domesticated animals).	Bathing or drinking water containing cercariae. Penetrative. Katayama disease.

NOTE.—Rare trematodes of man are: (1) Fasciola hepatica. Chiefly disease of sheep. Cercariae from snail (Lymnaea) encyst on grass. (2) Dicrocoelium lanceatum. Chiefly disease of cattle. (3) Metagonimus yokogawai. Another very small intestinal fluke of man. Intermediate hosts, mollusk and gold fish. (4) Echinostoma ilocanum. A rare intestinal fluke of the Philippines. (5) Two genera of Paramphistomoidea—Watsonius and Gastrodiscus. Intestinal flukes.

HELMINTHIC DISEASES (Continued). CESTODES

Parasite	Defin. host	Intermediate host	Important reservoir of virus	Transmission and pathogenicity
Diphyllobothrium latum.	Man.	1st, Cyclops strenuus and Diaptomus gracilis 2d, fish.	Man (faeces), dog and cat.	Eating raw fish containing plerocercoid larvae. Broad Russian tape-worm disease.
Hymenolepis nana.	Man.	Not required.	Children (faeces).	Man intermediate and definitive host. Ingestive. Dwarf tape-worm disease.
Hymenolepis diminuta.	Man and rat.	Rat fleas.	Rat.	Cases occur rarely in children. Probably ingestion of rat flea.
Taenia saginata.	Man.	Cattle.	Man (faeces).	Eating insufficiently cooked beef containing cysticerci. Ingestive. Beef tape-worm disease.
Taenia solium.	Man.	Hog.	Man (faeces).	Eating insufficiently cooked pork containing cysticerci. Ingestive. Pork tape-worm disease.
Echinococcus granulosus.	Dog.	Man, sheep and hogs.	Dog (faeces).	Dogs infected at abattoir. Hydatid disease.

NOTE.—Rare cestodes of man are: (1) Dipylidium caninum. A parasite of the dog with louse or flea as intermediate host. (2) Species of Davainea. Intermediate host possibly cockroach. These cestodes are probably accidental parasites of man.

In the table above, the only larval cestode of man given is *Echinococcus granulosus*. There are also two other larval cestodes reported for man which show the plerocercoid larvae of bothriocephaloid parasites. These parasites were originally placed in a collective group named **Sparganum.**

One of these parasites formerly known as *Sparganum mansoni* is now known to be the larval form of *Diphyllobothrium mansoni*. This parasite was first found by Manson at the autopsy of a Chinaman in 1882, and since then more than 50 cases have been reported. The adult parasite lives in dogs and cats. For further development the eggs must reach water where ciliated embryos develop in about 3 weeks. These gain entrance into the body cavity of a cyclops and develop into the procercoid larvae. Ciliated embryos then develop in 4 or 5 weeks. The plerocercoid stage develops in frogs which swallow the infected cyclops. These plerocercoid larvae, 5 to 15 inches long, are found coiled up in tumors of various parts of the body. How

the human infection is acquired is unknown. The parasites have been found in the ocular regions of man.

The other larval dibothriocephalid, *Sparganum proliferum*, has been found in one case in Japan and one case in Florida. These worms are quite small, not more than one-half inch in length, and are found in cysts in the skin. Nothing of the life history is known.

HELMINTHIC DISEASES (Continued). NEMATODES

Parasite	Defin. host	Intermediate host	Important reservoir of virus	Transmission and pathogenicity
Wuchereria bancrofti (Filaria bancrofti).	Man.	Mosquito (various species).	Man infected (blood).	Indirect in mosquito. Mature larva penetrates skin. Elephantiasis, etc.
Loa loa (F. loa).	Man.	Fly. Species of Chrysops (mangrove flies).	Man infected (blood).	Probably indirect (cyclical) in Chrysops. Probably inoculative. Ocular filariasis, etc.
Acanthocheilonema perstans (F. perstans).	Man.	Not definitely known.	Man infected (blood).	Transmission by mosquitoes and ticks suggested. No pathogenicity.
Dracunculus medinensis.	Man.	Species of cyclops.	Man infected (subcutaneous tissue).	Larvae enter cyclops. Infected cyclops in drinking water. Ingestive. Guinea worm infection.
Onchocerca volvulus (Filaria volvulus).	Man.	Not definitely known.	Man (blood?).	Possibly Glossina. Subcutaneous tumor and lymphangitis.
Strongyloides stercoralis.	Man.	Not required.	Man (faeces).	Parasitic filariform larva penetrates skin. Pathogenicity doubtful.
Necator americanus and Ancylostoma duodenale.	Man.	Not required.	Man (faeces).	Strongyloid larvae penetrate skin. Ancylostomiasis.
Trichinella spiralis.	Man (rat and hog).	Hog (man and rat).	Hog (muscle).	Encysted larva in raw or insufficiently cooked pork. Ingestive. Trichinosis.

NOTE.—Ascaris, Trichuris and Enterobius (Oxyuris) do not require intermediate hosts. With Ascaris and Trichuris, larva gradually develops in egg passed in faeces. Infection by ingestion of embryo-containing eggs. Embryo-containing eggs contaminate fingers from crushing female Enterobius in perineal region.

ARTHROPODAN DISEASES

Parasite	Life history	Disease and manifestations and remarks
Linguatula serrata (Linguatulidae).	Adult in nasal cavity of dogs, etc. Eggs in nasal mucus contaminate grass. Rabbits, cattle infected. Larvae in liver, lungs.	Porocephaliasis. Man may harbor adult or larva. Larvae usually in lungs or liver and do not seem to cause symptoms.
Armillifer armillatus (Linguatulidae).	Adults in lungs of snakes. Eggs contaminate water or food. Larvae in liver, lungs, etc., of lions, monkeys, rats, man, etc.	Porocephaliasis. Larvae wander in abdominal cavity or lungs in which latter they produce a chronic bronchitis which may resemble phthisis.
Demodex folliculorum (Demodicidae).	All stages passed within hair follicles or sebaceous cysts especially about nose. Adult may wander.	Demodectic acariasis. Causes a resistant itch in dogs. In man, may invade eyelids or Meibomian glands, and has been reported as causing various forms of dermatitis.
Sarcoptes scabiei (Sarcoptidae).	Female lives in burrow of skin giving off eggs which hatch into larvae.	Scabies. Burrows show as blackish lines, especially between fingers, flexor surfaces of arms and penis. Itching worse at night.
Pediculoides ventricosus (Pediculoididae).	Female lives on wheat straw worm or grain moth. Larval mites develop inside mother.	Grain itch. The mites leave wheat straw or grain and attack harvesters or those sleeping on straw mattresses. Attack upper trunk, neck and arms. Erythematous or vesicular eruption with constitutional symptoms.
Microtrombidium pusillum (Trombidiidae).	Adults live in fields or woods. The larval mite lives on grasshoppers or small rodents.	Autumnal erythema. The larval mites known as red bug or jigger attack man causing a severe itch.
Dermanyssus gallinae (Parasitidae).	Mites live in chicken houses and feed on fowls.	Poultryman's itch. The mites attack man producing eczematous dermatitis on backs of hands and forearms.
Glyciphagus domesticus (Tyroglyphidae).	Mites found in flour and sugar.	Grocer's itch.
Dermacentor venustus (Ixodidae).	Adults live on cattle, sheep, etc. May bite man.	Tick paralysis. The bite of this tick or Ixodes holocyclus may produce paralysis in sheep. An ascending type of paralysis due to tick bites has been several times noted in man, chiefly in children.
Pediculus vestimenti (Insecta, Pediculidae).	Adults live on clothing or hair and feed on man.	Pediculosis—Phthiriasis. Produce skin irritation with later on pigmentation (Vagabondismus).

ARTHROPODAN DISEASES (Continued)

Parasite	Life history	Disease and manifestations and remarks
Tunga penetrans (Dermatophilus penetrans) (Tungidae).	Impregnated female penetrates skin of man or animals.	Sandflea or chigoe infestation. Site of penetration shows as black spot with whitish induration surrounding it. Apt to form ulcers.
Dermatobia hominis (Oestridae: Bot Flies).	Eggs of fly become attached to mosquitoes or ticks. These latter bite man and larva penetrates skin.	A cutaneous myiasis. Larva at first club-shaped (ver macaque). Later worm-shaped (torcel). Larva causes a swelling with black opening. May invade eye.
Hypoderma lineata (Oestridae).	Larvae of this or other flies burrow under skin.	Creeping eruption. Larva migrans. The burrows make zigzag lines on face or soles of feet. Causes itching.
Chrysomyia macellaria, and C. dux (Muscidae). Oestrus ovis (Oestridae).	Fly deposits eggs in nostrils. Larvae wander to nasal sinuses. May invade aural canal.	Screw-worm infection. Nasal myiasis. Larvae in their wandering destroy tissues of nasal cavities, or of ear, and may cause death. May infest wounds or vagina. C. macellaria causes American nasal myiasis, C. dux causes Indian nasal myiasis and O. ovis causes African nasal myiasis.
Cordylobia anthropophaga (Ochromyia anthropophaga) (Muscidae).	An African fly; which deposits eggs on children and animals.	Larvae bore under skin, causing boil-like lesions with central opening. Larvae mature in two weeks.
Auchmeromyia luteola (Muscidae).	An African fly; deposits eggs on floors of native huts. Larvae bite man.	Congo floor maggot. The bite is not painful.
Calliphora vomitoria and Lucilia caesar and L. serricata (Muscidae).	Blow and green-bottle flies, depositing eggs on tainted meats. Larvae may be found in faeces.	Larvae may be cause of obscure abdominal conditions; may be found in nasal cavities, causing serious symptoms, or in ear.
Musca domestica (Muscidae).	Eggs presumably deposited near genitalia or ear.	Larvae have been found in male urethra and in the ear.
Sarcophaga carnaria (Sarcophagidae).	Viviparous. Larvae are deposited on decaying flesh (wounds, orifices of body).	Larvae gain access to wounds, nasal cavities, etc., at times causing death. Commonly found in intestinal myiases. May occur in vagina.
Anthomyia pluvialis (Anthomyidae).	Larvae deposited in body orifices.	Occasionally reported as found in ear.
Fannia canicularis (Anthomyidae).	Eggs deposited near external genitalia and larva penetrates urethra.	Symptoms of urinary irritation or obstruction. Has been found in gastrointestinal tract.

TABLE OF PARASITIC INFECTIONS OF MAN, ARRANGED ACCORDING TO COMMON LOCATION IN BODY. (*After Stiles*)

Appendix.—**Endamoeba*, †*Schistosoma*, *Ascarids, *hookworms, *pinworms, *whipworms.

Bladder.—†*Schistosoma*, **Sparganum*, **Anguillula*, **Agamomermis*.

Blood.—**Balantidium*, †*Trypanosoma*, *Schizotrypanum*, *malaria, **Plasmodium*, †*Hexathyridium*, †*Schistosoma*, **Microfilaria*, also young stages of various worms in transit.

Brain.—†*Paragonimus*, †*Schistosoma*, **Cysticercus*, †*Coenurus*, †*Multiceps*, **Sparganum*.

Dermal.—†*Leishmania*, *Mites (*itch, *hair follicle, *jigger, *straw itch, *ticks, etc.).

Ear.—**Otobius*, *spinose ear tick.

Esophagus.—**Gongylonema*, †*Physaloptera*.

External.—*Ticks, *Bed-bugs, *fleas, *lice, various insects.

Eye.—†*Agamodistomum*, †*Monostomulum*, **Cysticercus*, **Sparganum*, †*Agamofilaria*, †*Filaria*, †*Habronema*, †*Setaria*.

Generalized (more or less) somatic infections.—†*Leishmania*, **Rhinosporidium*, †*Paragonimus*, †*Schistosoma*, **Cysticercus*, **Echinococcus*, **Sparganum*, **Filaria*, **Trichinella*, †*Armillifer*, †*Linguatula*, **Porocephalus*.

Heart.—**Cysticercus*, *Dirofilaria*.

Intestine.—**Caconema*, †*Haemonchus*, **Heterodera*, †*Mecistocirrus*, †*Oesophagostomum*, †*Physaloptera*, **Rhabditis*, †*Ternidens*, †*Chordodes*, †*Gordius*, **Paragordius*, †*Echinorhynchus*, †*Macracanthorhynchus*, †*Moniliformis*, various insect larvae.

Intestine, large.—*Balantidium*, **Endamoeba*, †*Gastrodiscoides* †*Gastrodiscus*, †*Schistosoma*, †*Watsonius*, *Pinworms, **Oesophagostomum*, **Trichuris*, whipworm.

Intestine, small.—**Chilomastix*, **Councilmania*, †*Eimeria*, **Endamoeba*, **Endolimax*, **Giardia*, †*Isospora*, **Trichomonas*, †*Artyfechinostomum*, †*Clonorchis*, †*Echinochasmus*, †*Echinostoma*, †*Euparyphium*, †*Fasciolopsis*, †*Heterophyes*, †*Metagonimus*, †*Opisthorchis*, †*Stamnosoma*, †*Bertiella*, †*Braunia*, †*Davainea*, †*Diplogonoporus*, **Diphyllobothrium*, **Dipylidium*, †*Drepanidotaenia*, †*Hydatigera*, **Hymenolepis*, †*Ligula*, †*Raillietina*, **Taenia*, **Taeniarhynchus*, †*Ancylostoma*, *ascarids, *Enterobius*, *hookworms, **Necator*, *Oxyuris*, *pinworms, **Strongyloides*, *Syphacia*, *Toxascaris*, *Toxocara*, **Trichinella*, *Trichostrongylus*.

Kidney.—**Echinococcus*, †*Dioctophyme*.

Liver.—**Endamoeba*, †*Clonorchis*, †*Dicrocoelium*, †*Fasciola*, †*Opisthorchis*, †*Schistosoma*, **Echinococcus*, *Ascarids, †*Physaloptera*, †*Armillifer*.

Lungs.—**Endamoeba*, **Trichomonas*, †*Fasciola*, †lung flukes, †*Paragonimus*, *Hydatids, †*Metastrongylus*, also larval stages of *hookworms, **Strongyloides*, and *ascarids.

Lymphatic System.—**Filaria*, **Wuchereria*, also young stages of various worms in transit.

Mouth (cavity and membrane).—**Endamoeba*, **Trichomonas*, **Agamomermis*, **Gongylonema*, Insect larvae.

Muscles.—†*Sarcosporidia*, *Coenurus*, **Cysticercus*, †*Multiceps*, **Trichinella*.

Nasal Passage.—Thousand leggers, *screw-worms.
Pharynx.—†*Fasciola*, various worms in transit.
Stomach.—*Dicrocoelium*, *Fasciola*, †*Anguillulina*, †*Haemonchus*, also stages of intestinal parasites in transit.
Subcutaneous.—**Cysticercus*, **Sparganum*, **Agamofilaria*, †*Gnathostoma*, †guinea-worm, †*Lagochilascaris*, †*Loa*, †*Onchocerca*, †*Rhabditis*.
Vagina.—**Trichomonas*, †*Schistosoma*, †*Leptodera*, *pinworms.

* Contains species reported as indigenous, for man, in the United States or Canada.

† No species known as indigenous, for man, in the United States or Canada.

CHAPTER XVII

THE PROTOZOA

Classification of Protozoa

Class	Order	Genus	Species
Sarcodina (Rhizopoda). Motion usually by means of protoplasmic projections called pseudopodia.	Gymnamoebida	Endamoeba	E. histolytica
			E. coli
			E. gingivalis
		Endolimax	E. nana
		Iodamoeba	I. bütschlii
		Dientamoeba	D. fragilis
Flagellata (Mastigophora) Move by means of undulating membranes or flagella.	Monozoa	Trypanosoma	T. gambiense
			T. rhodesiense
		Schizotrypanum	S. cruzi
		Leishmania	L. donovani
			L. infantum
			L. braziliensis
			L. tropica
		Trichomonas	T. confusa
			T. vaginalis
		Chilomastix	C. mesnili
		Embadomonas	E. intestinalis
		Prowazekia	P. asiatica
	Diplozoa	Giardia	G. lamblia
Infusoria (Ciliata) These have contractile vacuoles and numerous fine cilia which are shorter than flagella and have a sweeping stroke.	Heterotrichida	Balantidium	B. coli
		Nyctotherus	N. faba
Sporozoa These have no motile organs. They live parasitically in the cells or tissues of other animals. Reproduction by spores.	Coccidiida	Eimeria	E. stiedae
			E. wenyoni
			E. oxyspora
		Isospora	I. hominis
	Haemosporidia	Plasmodium	P. vivax
			P. malariae
			P. falciparum
	Sarcosporidia	Sarcocystis	S. tenella

Classification of Protozoa (Continued)

Class	Order	Genus	Species
*Proflagellata (Spirochaetacea) Organisms transitional from bacteria to flagellates (Doflein).		Borrelia	B. recurrentis
			B. duttoni
			B. carteri
			B. novyi
			B. persica
		Leptospira	L. icterohaemorrhagiae
			L. icteroides
			L. morsus-muris
			L. hebdomadis
		Treponema	T. pallidum
			T. pertenue

* The Proflagellata are grouped with the protozoa table for convenience only and not to indicate taxonomic relationship. For discussion see page 436.

Note.—Hartmann and others have grouped the Haemosporozoa and the Haemoflagellata in an order Binucleata. The main characteristic is the possession of two differentiated nuclei, the kinetonucleus and the trophonucleus, at some developmental or transitional stage. While trypanosomes plainly show these characteristics, certain others, as the malarial parasites and the Leishman-Donovan bodies, having been modified as the result of cell parasitism, do not do so. This grouping together of the blood flagellates and sporozoa under the name Binucleata has been considered by many protozoologists as possibly convenient but not resting on sufficient ground to cause organisms with similar life histories as *Plasmodium* and *Coccidium* to be separated and the former to be placed with the blood flagellates in a new grouping.

It is a question as to whether the cellular bodies in the ascitic fluid of two cases in Berlin were really protozoa and to which the name *Leydenia gemmipara* was given. It has been considered that the protozoa classed as *Leydenia* were really abnormal *Chlamydophrys*. A protozoon, *Chlamydophrys enchelys*, has been found in fresh human faeces. This rhizopod has an oval hyaline shell with a terminal orifice from which filiform pseudopods project.

General Consideration of Protozoa

By the term protozoa we understand a branch of animals in which the individual is composed of a single cell morphologically and functionally complete; it is not one of a number of cells going to make up a complex individual and dependent on such a combination as is the case with the metazoa (there is no differentiation into tissues in protozoa).

Protista.—Recognizing the fact that certain protozoa have characteristics which make it impossible to draw a distinction between them and plants Haeckel has pro-

posed the name Protista as a designation for all simple and primitive living organisms whether they be plants or animals. In such a classification we would have the kingdom of Protista as well as the animal and vegetable kingdoms. In such a grouping the bacteria would be the lower types and the fungi and protozoal organisms the higher ones.

Cytoplasm.—Protozoal cells are made up of protoplasm which is divided into nucleus and cytoplasm. The cytoplasm is at times separated into an external, hyaline portion, the ectoplasm or ectosarc, and an internal granular portion, the endoplasm or endosarc. The functions of the ectosarc are protective, locomotor, excretory and sensory; those of the endosarc trophic and reproductive. Protozoa may be holozoic (animal-like) or holophytic (plant-like), saprophytic (fungus-like), or parasitic (living at the expense of some other animal or plant).

Nucleus.—The nucleus is characterized by concentration of the so-called chromatin substance of the cell. This chromatin however is usually combined with achromatin. The usually accepted test for chromatin is the staining affinity for basic aniline dyes. This test is now known to be unsatisfactory as other substances than chromatin may stain even more intensely. When chromatin is scattered through the cytoplasm, as extranuclear aggregations, such chromatin granules are called chromidia. There are cells where the chromidia take the place of the nucleus and from which a nucleus may be formed. Chromidia may arise from nuclei and nuclei from chromidia. The nucleus is made up of a network of linin, an achromatic reticulum in which is contained the nuclear sap or karyolymph. As a rule an achromatic nuclear membrane, continuous with the reticulum, separates the nucleus from the cytoplasm. In addition we have a substance which is achromatic (plastin) and which is the imbedding substance for chromatin grains. These plastin chromatin combinations are called karyosomes. The nucleoli are probably pure plastin. Plastin is to be regarded as a secretion or modification of chromatin made to serve as a matrix for the chromatin. Chromatin may be concentrated so that the nuclear space looks like a vesicle with a central chromatin mass (vesicular nucleus) or numerous chromatin grains may be scattered through the nuclear space (granular nucleus). The centrosome, which presides over cell division, is usually located just outside the nucleus. In some protozoa however the centrosome is within the nucleus and is often seen inside a karyosome being then called a centriole. The centrosome may also function over kinetic activities (flagellar motion) and is then termed blepharoplast.

When appearing as a small granule at the base of the flagellar apparatus it is called the basal granule. When there are extensions from it to the nucleus we have rhizoplasts.

Certain protozoa, as trypanosomes, show a differentiation of nuclei, the larger trophonucleus governing the functions of general metabolism and the smaller kinetonucleus directing the motor activities. Infusoria have a larger macronucleus which contains vegetative chromatin and a smaller micronucleus which contains reserve reproductive chromatin.

Reproduction.—Reproduction of protozoa may be by fission, when the nucleus and cytoplasm divide into two by simple division.

When the nuclei divide into a number of daughter nuclei, this nuclear division being followed by multiple division of the cytoplasm, we have sporulation.

Instead of fission we may have sexual reproduction or conjugation (zygosis). Here the nuclei of the separate sexual individuals (gametes) are termed pronuclei and the product of their fusion a synkaryon.

Where a single cell has division of its nucleus with subsequent fusion of these daughter nuclei to form a synkaryon the process is termed autogamy.

If two similar cells conjugate the term is isogamy; if dissimilar, as the macrogametes and microgametes of malaria, anisogamy.

The process of sexual union is termed syngamy and is of two kinds, (1) when the two gametes fuse completely or copulation and (2) when they remain separate and only exchange nuclear material or conjugation.

Organelles.—Protozoa have structures, termed organelles, that are concerned in the functions of locomotion, metabolism, etc. To those having to do with movement, the names pseudopodia, flagella, cilia and myonemes (contractile fibrils which give support to the body cells of certain protozoa) may be applied, and to those having to do with metabolism, food vacuoles and contractile vacuoles.

Sarcodina

The class Sarcodina is divided into the subclasses Amoebea and Proteomyxa, only the former concerning us medically. The Amoebea are creeping forms with branched root-like pseudopodia. The pseudopodia serve the double purpose of nutrition and locomotion. These protoplasmic extensions may be quite broad or very narrow—the lobose and the reticulose. The pseudopods do not anastomose. Sarcodina showing anastomosis of fine branching pseudopods are considered as Proteomyxa.

Some species of Sarcodina have hard shell-like coverings which are secreted in or on the ectosarc. These skeletons have openings through which the pseudopods project. The pseudopodia may be made up only of ectoplasm, or both ectoplasm and endoplasm may take part. Amoeboid movement always starts in the ectoplasm. In addition to the nucleus, which the so-called chromatin-staining method of Romanowsky brings out as reddish areas, or black with iron haematoxylin, we frequently observe aggregations of chromatin-staining material in the cytoplasm. These cytoplasmic chromatin bodies (chromidial bodies) are of importance in differentiating the encysted pathogenic amoeba from the nonpathogenic one. This extranuclear chromatin is supposed to play a part in the more intricate divisions which such protozoa undergo. Food vacuoles and contractile vacuoles are present in many species of Sarcodina.

Intestinal Amoebae

We now recognize a number of amoebae as parasitizing man, but of these one species only is generally admitted to be pathogenic (*Endamoeba histolytica*). The other species are of importance in differential

diagnosis but they do not seem to be associated with amoebic dysentery or its serious complication liver abscess. The more common non-pathogenic species are *Endamoeba coli*, *Endolimax nana*, *Iodamoeba bütschlii* and *Dientamoeba fragilis*.

The Pathogenic Amoeba.—Amoebae were first noted by Lambl, these being found in the stools of a child with diarrhoea in 1859. In 1875 Lösch described the parasite as found in intestinal ulcerations and

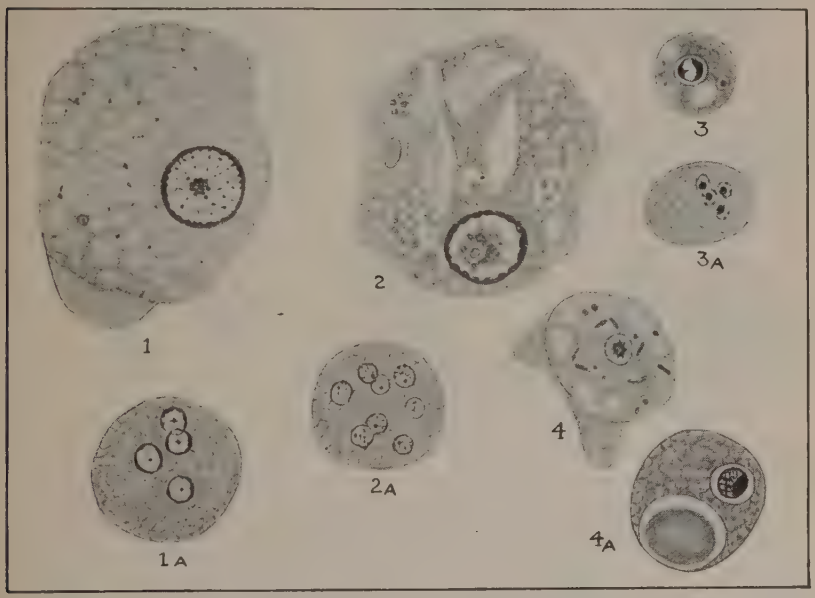


FIG. 92.—The more important intestinal amoebae of man showing nuclear structure when stained. 1. *E. histolytica*. 1(a). *E. histolytica* cyst. 2. *E. coli*. 2(a). *E. coli* cyst. 3. *E. nana*. 3(a). *E. nana* cyst. 4. *I. bütschlii*. (4a). *I. bütschlii* cyst. (*After Dobell.*)

stools of cases of chronic dysentery. In 1879 Grassi found encysted amoebae but as they were also found in the stool of well people he denied them a pathogenic rôle.

Koch and Kartulis noted the presence of amoebae in intestinal ulcers and the latter found them also in liver abscess.

In 1891 Councilman and Lafleur noted a pathogenic species, which was found in the submucosa of intestinal ulcerations, and also reported a harmless species.

In 1903 Schaudinn reported the existence of two species of amoebae, one harmless and named *Endamoeba coli*, the other pathogenic and named *E. histolytica*. In 1907

Viereck described a pathogenic amoeba which, by reason of its four nuclei in the encysted stage, he called *E. tetragena.*

As the result of the work of Hartmann, Whitmore, Darling, Wenyon and the conclusive findings of Walker we now hold the view that Viereck was working with

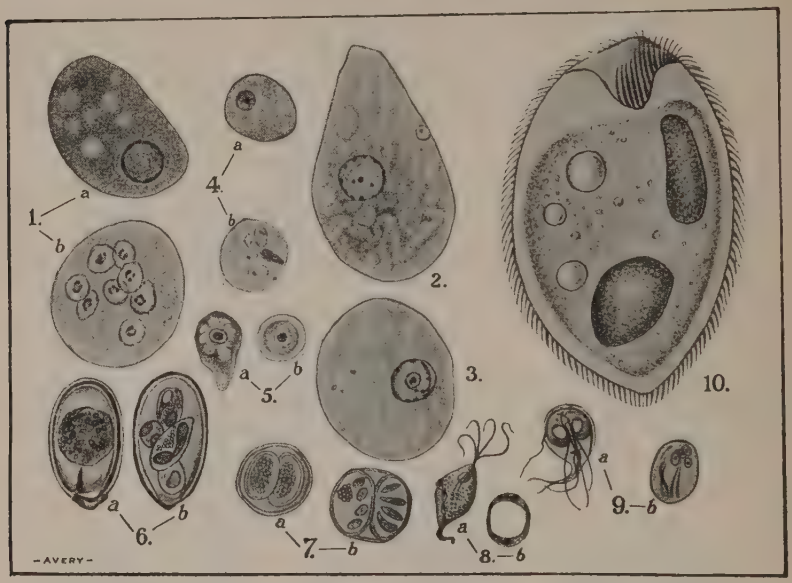


FIG. 93.—Important pathogenic protozoa of the intestinal tract. (1*a*) Motile *E. coli.* Note large amount and peripheral arrangement of chromatin in nucleus. (1*b*) Encysted *E. coli.* Note larger size than *E. histolytica* cyst, 8 ring-form nuclei and absence of chromidial bodies. (2) Motile *E. histolytica* from acute dysenteric stool. Note histolytica nucleus with scanty chromatin. (3) Tetragena type of *E. histolytica* from case of chronic dysentery. Note greater amount of chromatin and central karyosome with centriole. (4*a*) Preëncysted *E. histolytica* from carrier. Note small size and heavy peripheral ring of chromatin in nucleus, making this feature of chromatin in nucleus similar to the larger *E. coli.* (4*b*) Encysted *E. histolytica* from dysentery convalescent. Note small size, 4 ring nuclei and a dark chromatin-staining mass, "chromidial body." (5*a* and 5*b*) Motile and encysted cultural amoebae from Manila water supply. (6*a* and 6*b*) Oocyst and sporozoite production in 4 spores of *Eimeria stiedae.* (7*a* and 7*b*) Oocyst with 2 sporoblasts and oocyst with 2 spores containing 4 sporozoites of *Isospora hominis.* (8*a*) Vegetative *Trichomonas confusa.* (8*b*) *Blastocystis hominis.* (9*a* and 9*b*) Vegetative and encysted *Giardia lamblia.* (10) *Balantidium coli.* (*Illustrations of amoebae from Walker—others from Doflein.*)

E. histolytica and not with a separate species, so that by the law of priority we must drop the name *E. tetragena* and accept. *E. histolytica.* Some authorities prefer the generic names *Löschia* and *Entamoeba* to *Endamoeba.*

Schaudinn, in 1903, described the pathogenic amoeba, which he named *E. histolytica,* as follows: 1. Distinct, highly refractile and tenacious ectoplasm. He con-

sidered this tough external portion of the cytoplasm as the explanation of the ability of the pathogenic amoeba to bore its way into the intestinal submucosa. 2. Eccentric nucleus which was indistinct by reason of little chromatin. 3. Reproduction by peripheral budding in which small aggregations of chromatin reached the periphery of the cytoplasm and, enclosed in a resistant capsule, broke off from the parent amoeba and constituted the infecting stage.

We now know that Schaudinn's views as to reproduction are erroneous. The large, actively motile vegetative amoebae first divide into smaller precystic amoebae (*E. minuta*) which later encyst to give the characteristic cysts with four nuclei and chromidial bodies. These cysts when ingested by another individual develop in the intestinal tract into vegetative amoebae. Infection takes place only with cysts.

The pseudopods of *E. histolytica* are actively projected as long finger-like processes which show the ectoplasm quite distinctly. An important diagnostic feature for the pathogenic amoeba is the presence of red blood cells or tissue cells in the cytoplasm of the vegetative stage. The common nonpathogenic amoeba, *E. coli*, does not harbor red cells, but, instead, food particles or other debris.

For the differentiation of the various amoebae see accompanying tables.

FIG. 94.—*Endamoeba histolytica*. Mature cyst containing four nuclei and a mass of chromidial substance. (*After Hartmann.*)

Animal experiments.—Animal experimentation upon kittens with *E. coli* by Schaudinn, Craig and Wenyon have been unsuccessful as to production of dysenteric manifestations. On the other hand all of these experiments produced typical lesions and dysenteric manifestations in kittens injected rectally or fed with material containing pathogenic amoebae. Gastric juice tends to destroy vegetative amoebae.

Darling has been so successful in his experimental work with kittens that he compares the colon of a kitten to a test tube and suggests the procedure of rectal injections of material containing amoebae as a means of differentiating the two human amoebae.

On the other hand Walker was unable to infect kittens and monkeys with material containing pathogenic amoebae and he makes the statement that such failures would indicate the greater susceptibility of man to infection, as he was able to infect 17 out of 20 with one feeding of such material.

Wenyon produced a liver abscess in one of his experiments. In man the dislodgment of amoebae-containing material from amoebic intestinal ulcerations and the plugging of the portal capillaries by such emboli gives us the starting-point of a liver abscess. The exciting cause is *Endamoeba histolytica* which in the liver continues the same production of a gelatinous necrosis as is carried on in the submucosa of the large intestine or appendix.

Sellards and Baetjer note that the inoculation of kittens per rectum or by feeding dysenteric stools rich in amoebae has resulted in infection in about 50% of experiments.

By inoculating the material directly into the caecum they were able to infect every one of their kittens. They were also able to propagate a strain of amoebae through a series of animals for several months.

The intracaecal inoculations yielded positive results in diagnosis of human amoebiasis when the clinical manifestations were obscure and the amoebae in the discharges so few and atypical as to make such an examination unsatisfactory.

Human experiments.—Walker and Sellards carried on amoeba-feeding experiments with prisoners who volunteered their services.

The first series of experiments was with cultural amoebae, undertaken to refute statements that amoebae cultivated from water or vegetables, as well as from dysenteric stools, are capable of living in man parasitically or of producing dysenteric symptoms. Twenty feeding experiments on ten men were made by Walker and Sellards with cultures of amoebae without the development in a single instance of dysentery or the finding of such amoebae in the stools upon microscopical examination. In 13 cases they recovered the amoebae

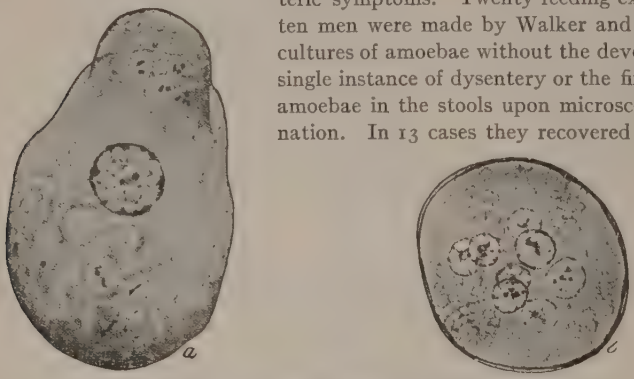


FIG. 95.—*Endamoeba coli.* *a*, Free amoeba; *b*, ripe cyst with eight nuclei. (*From Doflein after Hartmann.*)

in cultures from the faeces from the first to the sixth day, but never afterwards. They stated definitely that cultural amoebae are nonpathogenic.

The next experiments were with *Endamoeba coli.* In the 20 cases fed with material containing *Endamoeba coli* there was a uniform failure to recover them culturally and in no instance was dysentery produced. Seventeen became parasitized as the result of a single feeding in from one to eleven days, the endamoebae being found in the stools and persisting for extended periods. They concluded that *Endamoeba coli* is an obligate parasite, nonpathogenic, and cannot be cultured.

The third series of 20 feedings, carried on by Walker alone, was with *Endamoeba histolytica.* The material was mixed with powdered starch or magnesium oxide and given in gelatin capsules. In these experiments he obtained tetragena cysts in the stools of men fed only motile *Endamoeba histolytica*, and motile *Endamoeba histolytica* in the stools of men who were fed only tetragena cysts and, finally, an alternation of motile *E. histolytica* and tetragena cysts in the stools of a man having a recurrent attack of amoebic dysentery.

Seventeen of the men became parasitized after the first feeding; one required three feedings, and two, who did not become parasitized at the first feeding, were held as

controls. The average time for parasitization was nine days. Only 4 of the 18 parasitized men developed dysentery, which came on after twenty, fifty-seven, eighty-seven, and ninety-five days, respectively, after the ingestion of the infecting material.

In 4 cases fed with material from acute dysenteric stools or from amoebae-containing pus from liver abscess, and containing motile amoebae, there was no resulting dysentery, the 4 cases of experimental dysentery resulting from feeding of material from normal stools of carriers.

As regards the cases which became parasitized, but did not develop dysentery, it is suggested that the amoebae live as commensals in the intestine of the host and penetrate the intestinal mucosa and become tissue parasites only when there occurs depression of the natural resistance of the host or as the result of some lesion of the intestine. That the pathogenic amoebae are more than harmless commensals, however, is shown by the fact that they alone, and not the nonpathogenic *Endamoeba coli*, are capable of penetrating a possibly damaged intestinal mucosa.

Epidemiology.—The old idea that water, fruit or vegetables, from which one can isolate amoebae upon culture, are sources of infection must be abandoned, as such cultural amoebae are known to have no pathogenic relation to man.

The chief factor in the spread of amoebic dysentery would seem to be the encysted amoebae in the stools of convalescents or healthy carriers rather than the motile ones in dysenteric stools. This probably explains the endemic rather than epidemic characteristics of the spread of amoebic dysentery because if the innumerable vegetative amoebae in dysenteric stools were equally operative with the more sparsely eliminated cysts there would be epidemics of amoebic dysentery similar to those of bacillary dysentery.

Our present view is that the carrier is the chief factor in the spread of amoebic dysentery and when such an individual has to do with the preparation of food he becomes a particular source of danger.

Vegetative amoebae undergo disintegration in a short time after the stool is passed, so that they are probably rarely concerned in amoebic infections but the resisting cysts may be washed from a dried stool into a water supply or even be transported in dust to lodge on unprotected foodstuffs.

Flies may possibly act as transmitting agents. As bearing on the probable importance of such flies as *Musca domestica* and *Fannia canalicularis* in transmitting amoebic infections may be noted the findings of Wenyon that the faeces of such flies, as well as *Lucilia* and *Calliphora*, after feeding on cyst-containing human faeces, teem with such cysts.

Geographical distribution.—Amoebic dysentery seems to be especially prevalent in Indo-China, China and the Philippines, as well as in parts of India. It is also very common in Egypt and Northern Africa. In South America, especially Brazil, it is

common, as is also true of the West Indies and Central America. It is an important disease in the Southern States of the United States, as well as in Italy and other parts of Southern Europe. On the whole it is probable that it exists in greater or less degree in most of the tropical and subtropical parts of the world and is more common than is generally believed to be the case in the temperate regions.

In the civilian population of England the estimate of healthy cyst "excretors" is 3%, and in France 5%. In an examination of the stools of 13,043 Americans, Stiles found 333 (4.1%) to show *E. histolytica* infection.

Laboratory diagnosis.—The mucoid mass of amoebic dysentery is often brownish. The pathogenic amoeba shows active finger-like processes and in acute attacks often shows contained red cells. In the fresh specimen of the milky mucopurulent mass of bacillary dysentery one observes large numbers of pus cells and particularly very large phagocytic cells which greatly resemble amoebae. Upon staining with Gram's stain one may find numerous Gram-negative bacilli in the cytoplasm of this cell.

These large cells which resemble amoebae are often vacuolated, thus intensifying the similarity. They are nonmotile, however, and do not show the small ring nucleus which is so characteristic of the vegetative human amoebae. The nucleus of the confusing cells is also larger, approximating one-fourth the size of the cell.

For bringing out the nuclear characteristics of human amoebae Walker recommends fixation of thin moist smears in Giemsa's sublimate alcohol (absolute alcohol 1 part, sat. aq. sol. bichloride 2 parts) for ten to fifteen minutes. These smears are then well washed with water and stained with alum haematoxylin for five minutes. The nuclear characteristics have been noted previously.

With vegetative amoebae I have obtained beautiful results with vital staining which can best be done by tinging the faeces emulsion with a 1% aqueous solution of neutral red.

I have also had good results by emulsifying the faeces in a drop of 1 or 2% formalin and then adding a drop of 2% acetic acid. The mixture is then tinged with either neutral red or methyl green.

For distinguishing the encysted form of *Endamoeba coli* one can obtain excellent results by emuls fying the faeces in Gram's iodine solution. Owing to the glycogenic reaction given by *E. coli*, the round amoeba, with its 8 nuclei, stands out very distinctly. At present we are using the highly recommended iodine-eosin method. See page 66.

For diagnosing the 4-nucleated cyst of the pathogenic amoeba one gets better results with haematoxylin as this brings out not only the 4 nuclei but the chromidial bodies as well.

Dobell states that chromidial bodies are not particularly characteristic of the cysts of *E. histolytica*. He does, however, note, that with *E. histolytica* they occur as fairly large blocks or bars, with rounded ends when fully developed; while with *E. coli* they are acicular or filamentar, and often aggregated into sheaves, resembling fragments of splintered glass.

It was formerly customary to recommend the administration of salts prior to examining for amoebae. Walker warns that such a procedure gives us amoebae which are difficult to differentiate, the nuclear characteristics of *E. coli* and the tetragena nucleus of *E. histolytica* being much alike as they both contain much chromatin. In a dysenteric stool the histolytica type of nucleus, containing but little chromatin, does not resemble the nucleus of *E. coli*.

He prefers the examination of formed stools obtained without a purgative; and notes the advantages of examining a specimen with a ⅔-inch objective as encysted amoebae are then easily perceived.

The preëncysted *E. histolytica* has a nucleus much resembling that of *E. coli*. The presence of the same chromidial bodies one notes in the cysts is an aid in recognizing this stage. The 4 nuclei of the cysts are much smaller than the nucleus of the preëncysted and vegetative stages.

Wenyon and others have recently emphasized the difficulties of differentiating human amoebae in the vegetative stage, and consider the criterion of presence of red cells in the amoebae as the sole valid one indicating the pathogenic amoebae. With a dysenteric stool containing amoebae, but no phagocytized red cells, the probability is that it is a stool of bacillary dysentery. A helpful method of differentiating amoebae is examination of the cysts and as these alone are of practical importance in infection only such forms need be considered in carrier examinations. Definite differentiation of the various human amoebae, however, depends on the staining characteristics of their nuclei. See tables page 402. For staining methods see page 65.

Cultivation of Amoebae.—Until quite recently it was believed that the parasitic amoebae of man were not cultivable. As a matter of fact it is now generally accepted, that prior to the work of Cutler, Boeck and Drbohlav, Thomson and Robertson and others, that all amoebae cultivated belonged to free-living species. Cutler in 1918 reported the cultivation of *E. histolytica*, but his work was not confirmed. In 1924 Boeck, while culturing a stool for flagellates, using Locke-egg-serum medium, definitely cultured the *E. histolytica*.

Since that time Boeck, Drbohlav and others, using Locke-egg-serum medium and Locke-egg-albumin medium have been successful in cultivating various species of amoebae. Craig and St. John claim to have had success with more simple media and state that they have found cultural methods superior for diagnostic purposes to

either the sedimentation method or direct examination of faeces, particularly when only one microscopical preparation is examined. For preparation of media see page 50.

Method of inoculating media.—Place a loopful of the faeces in the medium (warm) and break up thoroughly. Incubate at 37°C. for twenty-four hours. The amoebae will be found in the sediment at the bottom of the culture. Transfers should be made within forty-eight hours using a pipette.

The Nonpathogenic Amoebae

Endamoeba coli.—This, the largest of the human amoebae (20 to 40μ), was described by Schaudinn as follows: (1) No distinction between a granular endoplasm and refractile ectoplasm; (2) centrally placed and sharply outlined nucleus, rich in chromatin, and (3) formation of eight-nucleated cysts which form the infecting stage.

The cytoplasm of the vegetative organism never shows ingested red cells but, instead, food vacuoles containing bacteria, starch grains, etc. The pseudopods, which are lobose and greyish, are sluggishly projected. Before encystment the vegetative forms divide into smaller ones from which the eight-nucleated cysts develop. The cysts are round or oval and vary from 12 to 20μ in diameter. They do not show the large chromatoid bodies so characteristic of the *E. histolytica* cyst and also have a thicker cyst wall. They stain deeply with iodine stains.

This amoeba is quite common in well individuals as well as in those presenting various symptoms of disease. The incidence ranges from 15 to 25% in examinations of the stools of soldiers. Kofoid gave 23% for American soldiers.

Endolimax nana.—It is a remarkable fact that one of the most common of the intestinal amoebae, *E. nana*, has only recently been reported (Wenyon and O'Connor in 1917). In the examination of the stools of American soldiers Kofoid found it present in 28% of his examinations. In examining 156 British soldiers Dobell noted its presence in 33% of them. The general view is that it is not pathogenic but its great importance lies in the possibility of its being mistaken in its cyst form for *Endamoeba histolytica*. The living amoeba averages about 8 microns but in stained specimens it is somewhat smaller. In freshly passed faeces the amoeboid motion is sluggish and the nucleus indistinct. This amoeba is best identified by haematoxylin-stained specimens when the nucleus shows a measurement of about 2 microns and the collection of all chromatin in a large, variously shaped karyosome—the limax-type of nucleus. Unless perfectly fresh material is stained the degenerative changes in the *Endamoeba* may give a signet ring appearance of karyosome and ring nucleus. Such an appearance may suggest the limax nucleus. The mature cysts are usually oval, but sometimes round, and contain 4 nuclei, but when newly formed may have only one nucleus. The nucleus is large in the uninucleate form (up to 3 microns) but in the quadrinucleate forms it is about 1.2 microns with a large eccentric mass of chromatin. Chromidial bodies are rarely, if ever, present in these cysts.

Iodine cysts.—These bodies which stain a deep brown color when treated with iodine are now recognized as amoebae and have been named *Iodamoeba bütschlii*. They are most probably nonpathogenic. It is a small amoeba (9 to 13 microns, rarely up to 20 microns) and shows the sluggish movements of *Endamoeba coli*. The nucleus is very difficult to discern, thus differing from *E. coli*. In stained speci-

mens it has a vesicular nucleus about 2 microns in diameter with a fairly large central karyosome. The cysts are spherical or oval, often of irregular outline and are about 10 microns in diameter. The nucleus is large and eccentrically placed and has a karyosome which tends to show as a peripherally placed mass. There is almost always present a large glycogen mass in the cyst which stains intensely with iodine.

Dientamoeba fragilis (Jepps and Dobell 1918).—A rare binucleate amoeba averaging 8–9 microns in size. Nuclei show a fairly large, central granular karyosome and no peripheral chromatin. Strands of linin may be seen radiating from karyosome to nuclear wall. This organism is said to be frequently mistaken for *Blastocystis* when the vacuoles coalesce leaving a thin ring of cytoplasm. Cysts unknown. Considered nonpathogenic.

Kofoid and Swezy reported another species of amoeba parasitic in man, *Councilmania lafleuri*. The adult and cystic stages resemble in many respects the *E. coli*. Adult said to ingest red blood cells. Stated to be pathogenic but that more evidence is needed on this point.

Caudamoeba sinensis has been reported by Faust as the etiological agent of a dysentery in China.

Endamoeba gingivalis varies from 10 to 25μ in diameter. The nucleus is much smaller than those of the intestinal amoebae and has a distinct nuclear membrane enclosing a deeply stained karyosome. On the whole it is poor in chromatin, in this respect rather resembling the histolytica nucleus. Pseudopod projection is less active than that of *E. histolytica* but more so than *E. coli*. Encysted forms are very rarely seen and these do not show evidence of reproduction.

This amoeba formerly considered important in the etiology of pyorrhea alveolaris is now generally regarded as a saprophytic organism living along with the numerous spirochaetes and bacteria in the mouth especially in pockets resulting from suppurative conditions.

Key to Genera and Species of Amoebae (Dobell and O'Connor)

1. (*a*) One nucleus present in active amoeba 2
   (*b*) Two nuclei present ..... Genus *Dientamoeba* 6
2. (*a*) Nucleus with small spherical karyosome and peripheral layer of fine chromatin beads ..... Genus *Endamoeba* 3
   (*b*) Nucleus with large irregular eccentric karyosome and no peripheral chromatin granules ..... Genus *Endolimax* 4
   (*c*) Nucleus with large central spherical karyosome surrounded by a layer of achromatic granules ..... Genus *Iodamoeba* 5
3. (*a*) Ripe cyst, 4 nuclei; glycogen diffuse; large chromatoids generally present ..... *E. histolytica.*
   (*b*) Ripe cyst, 8 nuclei: glycogen in early stages only; large chromatoids occasionally present but often absent ..... *E. coli*
4. Ripe cyst, 4 nuclei; glycogen rarely present; chromatoids absent ..... *E. nana.*
5. Ripe cysts, 1 nucleus; glycogen in a dense mass; no chromatoids ..... *I. bütschlii*
6. Nuclei with central granular karyosomes and no peripheral chromatin. (Cysts unknown) ..... *D. fragilis.*

DIFFERENTIATING CHARACTERISTICS OF PARASITIC AMOEBAE (AFTER DOBELL AND O'CONNOR).

MOTILE STAGE

	Endamoeba histolytica	Endamoeba coli	Endolimax nana	Iodamoeba bütschlii
Size.....	20–30μ	20–30μ	6–12μ	9–13μ
Motility.....	Characteristic. Freshly removed from host displays astonishing activity. Flows in almost straight line across field. Soon becomes less active pushing out a few large, blunt, blade-like pseudopodia which are perfectly hyaline being composed entirely of ectoplasm. This movement may continue for hours before amoeba rounds up and dies.	Freshly removed may show considerable activity, as a rule, however, extremely sluggish with little locomotory movement. Motions consist chiefly in changing of shape without evident progression. Formation of large, clear, blade-like pseudopodia never seen. Degenerate, motionless or dead forms frequently indistinguishable from similar forms of *E. histolytica*.	As a rule not very active. Has slow progressive movement in fresh preparations. Later shows no movement save change of shape. Pseudopodia few, blunt and thick. Soon rounds up and dies and this more or less degenerate form is the one most commonly seen in stools.	Generally but slightly motile. Movements similar to *E. coli*. Greatly resembles when alive small specimens of *E. coli*. Quickly degenerates and dies.
Cytoplasm.....	Endoplasm colorless, finely granular, and uniform in appearance. May contain red blood cells and fragments of host's tissue cells, but bacteria and other particles in host's faeces probably never ingested normally. Ectoplasm clear and well developed with sharp line of demarcation separating it from the endoplasm.	Endoplasm bulky, granular and usually contains numerous food vacuoles charged with bacteria, yeasts, vegetable débris and other particles derived from host's faeces. Red blood cells not ingested. Other vacuoles, spindle-shaped, containing liquid seen. No sharp line of demarcation separates the ectoplasm from the endoplasm.	Displays few noteworthy features. Endoplasm finely granular with numerous minute food vacuoles containing ingested bacteria. Red blood cells not ingested. No sharp line of division distinguishable as a rule between the endoplasm and the ectoplasm.	Displays few peculiarities. Endoplasm finely granular and homogeneous in appearance. Usually contains numerous food vacuoles charged with minute bacteria. Red blood cells not ingested. Cysts of this amoeba have been previously described as "Iodine cysts."
Nucleus.....	4–7μ.—Unstained a delicate vesicle inconspicuous or invisible. Stained shows fine chromatin granules lining wall giving a finely beaded ring appearance. Karyosome small, spherical and usually central. Chromatic part 0.5μ in size.	4–7μ.—Unstained readily distinguishable. Stained shows larger beads of chromatin lining wall. Karyosome, spherical, usually eccentric and larger than that of *E. histolytica*. Chromatic part 1μ in size.	1–3μ.—Stained the nucleus is vesicular with a delicate membrane free from chromatin. All chromatin contained in a large, irregular, eccentrically placed karyosome. Variations in size and shape of karyosome characteristic of this species.	2–3.5μ.—Unstained readily distinguishable as a small vesicle with a distinct membrane. Stained shows wall free from chromatin as a rule. Typically chromatin almost entirely contained in a large, central, spherical karyosome. Zone between nuclear wall and karyosome filled with single layer of small granules.

PRECYSTIC STAGE

	Endamoeba histolytica	Endamoeba coli	Endolimax nana	Iodamoeba bütschlii
	Active amoebae in lumen of gut undergo division leading to decrease in size. Therefore, these forms vary in size. Red cells and other food particles expelled. By secreting delicate, transparent wall become cysts. Nucleus structurally similar to that of adult.	Formation same as with *E. histolytica*. Often indistinguishable from *E. histolytica*. In fact degenerate forms of either species can never be differentiated with certainty and lead to error in diagnosis. Nucleus structurally similar to that of adult.	Unlike species of *Endamoeba* this stage is not smaller than the adult. Differs only in not containing food inclusions. Nucleus structurally same as that of adult.	Same size as adult. No food inclusions and the cytoplasm is clear and glassy white. Nucleus differs from adult in containing more granules in clear zone between karyosome and nuclear wall.

ENCYSTED STAGE

Size	7–15μ	15–20μ	7–9μ	9–12μ
Shape	Round.	Round.	Usually oval.	More or less rounded.
Wall	Thin.	Thicker than *E. histolytica*.	Thin.	Relatively thick.
Nuclei	Typically shows four nuclei. May show one to four. Nuclei at rest structurally similar to that of adult nucleus.	Typically shows eight nuclei. May show one to twenty. Structurally similar to adult nucleus.	Very small. May show from one to four nuclei. Rarely eight. Structurally similar to adult type of nucleus.	One relatively larger nucleus. Differs structurally from the adult type in that the granules in the clear zone become massed at one pole giving an eccentric karyosome.
Chromatoids	Large chromatoids with blunt, rounded ends common.	Large spicular chromatoids may be present but usually absent.	Absent.	Absent.
Glycogen	Diffuse but not abundant.	Relatively abundant in the early stages. Scanty or absent in mature cysts.	Rarely present.	Dense glycogen mass is characteristic.

Flagellata (Mastigophora)

In this class of protozoa the adults have flagella for the purposes of locomotion and the obtaining of food.

Some flagellates more or less resemble rhizopods in being amoeboid and in having an ectoplasm and an endoplasm. The body is frequently covered by a cuticle (periplast). Some flagellates have a definite mouth part, the cytostome, which leads to a blind oesophagus; others absorb food directly through the body wall. In addition to flagella, some flagellates possess an undulating membrane. All flagellates possess a nucleus and some have contractile vacuoles. The flagellum may arise directly from the nucleus or from a small kinetic nucleus, the blepharoplast (micronucleus or basal granule).

The most important flagellates of man are the haemoflagellates, *Trypanosoma* and *Leishmania*. In addition we have flagellates in the intestinal canal and in the vaginal secretion. Some authors place the genus *Piroplasma* with the flagellates and there has been controversy concerning the nature of certain projections from these bodies. It would seem preferable, however, to consider them under the Sporozoa.

The Trypanosomidae.—This family according to Wenyon includes (a) the *Trypanosoma* which are parasites of blood of vertebrates or invertebrates (b) the *Leptomonas*, *Crithidia* and *Herpetomonas* which are intestinal parasites of invertebrates; (c) the *Leishmania* which are parasites of vertebrates and invertebrates; and (d) the *Phytomonas* which are parasites of invertebrates and plants.

The Trypanosomidae possess a nucleus and a single flagellum arising from the blepharoplast which is part of the kinetoplast. The body is an elongated, flattened and curved blade of cytoplasm, somewhat tapering at each end.

The simplest type is the leptomonas, with the kinetoplast near anterior end.

In the crithidia type, the kinetoplast is near but still anterior to the nucleus. The axoneme of flagellum passes from kinetoplast to convex margin of the body and then along its surface or on the edge of a cytoplasmic ridge (the undulating membrane) to anterior end of body and becomes the flagellum. The free margin of such a membrane is longer than the attached margin, hence membrane is thrown into folds, and when functioning has undulatory movement.

In the trypanosome form the kinetoplast is posterior to the nucleus, the axoneme passing from the kinetoplast to anterior end of body in same manner as in the crithidia type. Either *Leptomonas*, *Crithidia*, or *Trypanosoma* may transform to produce shorter and more rounded individuals, the leishmania form, which contains nucleus and kinetoplast in a small rounded body in which the axoneme extends from kinetoplast to edge of body.

The leishmania form will transform again under proper conditions to any one of the flagellate types from which originally derived.

The genera *Leishmania* and *Leptomonas* exist only in the leishmania and the leptomonas form. The genus *Crithidia* exists in addition to the above two forms in crithidia form, while the genera *Herpetomonas* and *Trypanosoma* can exist in any of the three forms and in addition in the trypanosome form.

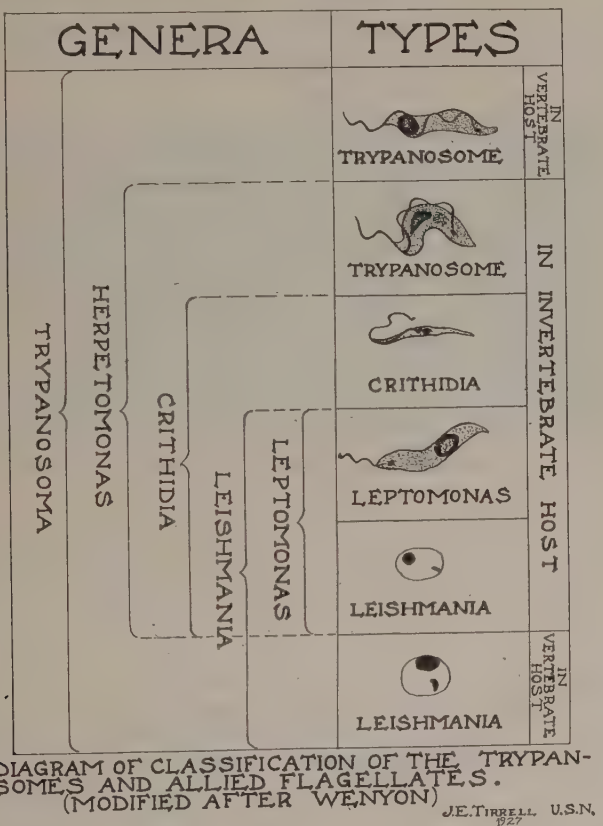


FIG. 96.—Trypanosomidae.

Trypanosomes of Sleeping Sickness

Trypanosoma gambiense and T. rhodesiense.—The African trypanosomiases follow infection with two species of trypanosomes; the more virulent type of the disease, occurring in South Central Africa, being due to *Trypanosoma rhodesiense*, transmitted by *Glossina morsitans*

and that of less severe type, but of more general distribution, being due to *T. gambiense* and transmitted by *Glossina palpalis*. The very important *Trypanosoma brucei*, which is the devastating agent in the African horse, dog and cattle disease, nagana, is also transmitted by *Glossina morsitans* and there exists the opinion that this trypanosome is identical with *T. rhodesiense*. A third human trypanosome is *T. nigeriense*, said to be transmitted by *G. tachinoides*. This trypanosome is generally considered as identical with *T. gambiense*.

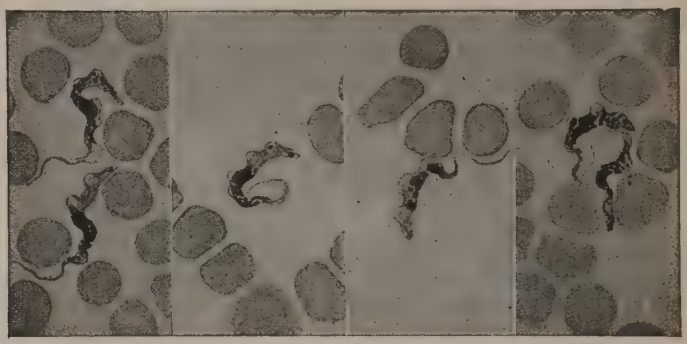


FIG. 97.—*Trypanosoma gambiense* (slide presented by Professor F. G. Novy). (*From Todd.*)

Castellani considers *T. gambiense* as representing two trypanosome infections. The one which gives rise to a more chronic course and is less virulent for animals he terms *Castellanella gambiensis*. That which gives rise to a more acute form of disease and is more virulent for laboratory animals is recognized as *Castellanella castellanii*.

These trypanosomes are blood flagellates and are typical of the Binucleata in possessing two chromatin-staining areas, the larger and more centrally situated mass being the tropho or macronucleus and the smaller, but more deeply staining one, the kineto or micronucleus (blepharoplast). Trypanosomes have a fusiform or fish-shaped body which stains blue. Near the less pointed, nonflagellated end, usually called the posterior end, is the deeply stained blepharoplast. Behind this is a vacuole and, taking origin from a minute basal granule near the blepharoplast is the flagellum. This borders an undulating membrane attached to the body and then, carried along to the other extremity, projects free as a long, whip-like flagellum.

In fresh preparations the body of the trypanosome progresses in the direction of its flagellated end, although occasionally it will be observed to move in the opposite direction.

T. gambiense varies much in length and breadth. The normal type, as found in the blood, varies from 14 to 20 microns, while longer forms, 20 to 24 microns, are growth ones and, in the longest ones (23 to 33 microns), we have those preparing

to divide longitudinally. The normal short forms are the ones from which the development takes place in the tsetse fly. In width these flagellates are from 1.5 to 2 microns. The blepharoplast is oval and the nucleus situated at about the center.

With *T. rhodesiense* the nucleus is typically located almost adjacent to the blepharoplast. As a matter of fact, passage of this trypanosome through rats is required to bring out these "posterior nuclear forms," the nuclear location in man being almost entirely that of *T. gambiense*. Even in experimentally infected rats, not more than 5 or 6% show posterior nuclear forms. With *T. rhodesiense*, infection of laboratory animals runs an acute course to death; with *T. gambiense*, inoculation, which is often without result, will if successful produce an infection running a chronic course.

When the tsetse fly, *Glossina palpalis*, feeds on a man in whose peripheral circulation there are normal type trypanosomes we have an accumulation of such forms in the middle and posterior portions of the gut. From the eighth to the eighteenth day long, slender forms develop and pass forward into the proventriculus. None of the intestinal forms can cause infection when injected into animals. These proventricular types work their way into the salivary ducts and thence into the salivary glands, where further development takes place. Here we have shorter forms developing, which are similar in morphology to the normal blood type. It is at this stage that the fly becomes infective by the passing of these trypanosomes down the salivary ducts and through the channel in the hypopharynx to the subcutaneous tissues of the person bitten. High temperatures, 75 to 85°F., are favorable to extracorporeal development, while low temperatures, 60 to 70°F., are inimical to development, but do not kill the ingested trypanosomes. This explains the long period which at times elapses before a fly becomes infective. Under favorable conditions a fly becomes infective in twenty to twenty-four days and remains infective the rest of its life, up to 185 days. The infection is not transmitted to the pupa. This is an inoculative, cyclical or indirect type of infection. It is usually considered that a tsetse fly whose proboscis has just been contaminated with trypanosome blood is capable of transferring the infection for a few hours. This would be a mechanical or direct method of infection and such power for infection lasts only for a few hours.

When tsetse flies feed on animals infected with trypanosomes only from 2 to 6% become infective. Again, it has been shown that where the wild animals on which tsetse flies feed may show an infection of from 16 to 50% yet not more than two out of every 1000 tsetse flies, caught and tried out on susceptible animals, show themselves infective.

Both of the human trypanosomes of Africa have been cultured by using the N.N.N. medium in which rat's blood was substituted for that of the rabbit. Human blood will also serve as a substitute. Growth however is not constant.

For the laboratory diagnosis we may use peripheral blood with some thick-film method. The examination of preparations from the periph-

eral blood is usually very discouraging. Very much better results (in fact some prefer this method to any other) can be obtained by taking 10 to 20 cc. of blood into about 25 cc. of citrated salt solution, centrifuging two or three times and examining the sediment of the third centrifugalization. Dutton and Todd prefer to centrifuge citrated blood and to collect the leukocyte layer for examination as is done in opsonic work.

The English workers usually prefer the gland puncture method, using a sterile but dry hypodermic needle. Water in the needle distorts both Leishman bodies and trypanosomes.

In the sleeping sickness stage trypanosomes can almost constantly be found in the cerebrospinal fluid.

Some prefer to inoculate susceptible animals, particularly the guinea pig or monkey, with blood or gland juice from the suspected case. A very satisfactory material is an emulsion from an excised gland which may be inoculated intraperitoneally into white rats. The further course, after animal inoculation, is the examination of the blood of these animals for trypanosomes. Usually at the time the guinea pigs die we find numerous trypanosomes.

Other tests are: (1) Trypanolysis when unheated suspected serum and trypanosomes are incubated together for one hour. Normal serum may occasionally cause disintegration and treated cases give it in only about 45% of cases. Unfavorable, untreated cases give it in about 80% of cases.

(2) The so-called autoagglutination test is not of much value. In this the red cells of the blood of a trypanosomiasis case come together in clumps when one makes a wet preparation. It is not a rouleaux formation. (3) The attachment test is made by making a mixture of inactivated serum, leukocytes and trypanosomes and allowing them to be in contact for twenty minutes. A positive test shows attachment of the trypanosomes to the leukocytes.

Trypanosomes of Animals

Of the more important pathogenic trypanosomes of animals may be mentioned:

Trypanosoma brucei.—This trypanosome causes a disease surely fatal in horses and one from which few cattle recover. It is called "nagana" or the fly disease, from being transmitted by the tsetse fly, *Glossina morsitans*. All animals except man and possibly the goat seem susceptible. The disease is characterized by fever, oedematous areas about the neck, abdomen and extremities, progressive anaemia and emaciation. It is an important disease of domesticated animals of many parts of Africa.

Trypanosoma evansi.—This is the cause of a very fatal disease of horses in India and the Orient known as "surra." It also affects camels and even cattle. It is thought to be transmitted by biting flies (*Stomoxys*). The symptoms are fever, emaciation, oedematous areas and great muscular weakness.

Trypanosoma equinum.—This trypanosome causes a fatal disease in horses in South America. There is paralysis of the hind quarter of the horse which gives the disease the name "mal de caderas."

Trypanosoma equiperdum.—This trypanosome causes a disease of horses in many parts of the world. It is known as "dourine" and is transmitted by coitus. The genital organs show marked oedema which is followed by anaemia and paralysis.

Trypanosoma dimorphon.—This trypanosome causes a disease of horses in Gambia. It is also found in horses and cattle in other parts of Africa. The parasite shows marked variation in morphology.

Trypanosoma lewisi.—Rats in many parts of the world show this infection which is rarely fatal to them. It is transmitted by the rat flea by a process of regurgitation. It can also be transmitted by the rat louse.

There are many trypanosomes in birds, frogs, fish, etc.

The Trypanosome of Chagas' Disease

Schizotrypanum cruzi (Trypanosoma cruzi).—In 1909, Chagas reported the finding of a flagellate in the intestines of *Conorhinus megistus* or, more properly, *Triatoma megista*. He was also able to transmit the flagellate to laboratory animals and could culture it on blood agar.

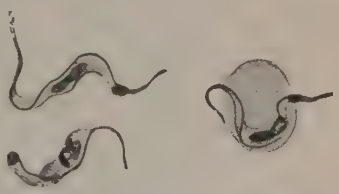


FIG. 98.—*Schizotrypanum cruzi* in blood of child with acute type of Brazilian trypanosomiasis. (*MacNeal from Doflein after Chagas.*)

In investigating the matter of the importance of this flagellate, *Schizotrypanum cruzi*, in Minas Geraes, Brazil, where the bug was present in great numbers in the cracks of the houses of the poor, he associated this flagellate infection, which he at first considered trypanosomal, with a disease of the children of that section.

The bug is a vicious feeder and, from its biting chiefly about the face, has been called *barbiero* or barber by the natives. Both the male and female of *Triatoma* bite and can transmit the disease and although the parasite is not transmitted hereditarily the nymph is capable of sucking blood and becoming infected.

It requires several months for the insect to go through the egg, larval and pupal stages to maturity. The insects may live for more than a year and tend to remain in the same house where they may have become infected but leave such house if it be abandoned by man. Brumpt thinks that the bedbug also may transmit the disease and has shown that *Rhodnius prolixus*, a bug common in Venezuela, is able to do it. Ticks have also been incriminated.

The parasite.—*S. cruzi* is found in the blood of children during the acute febrile stage but at other times in children, and as a rule in adults, it is rarely present in the peripheral blood. The early blood forms are narrow and very motile. They increase in size and slacken in motility when they become about 20 microns long. *S. cruzi* is characterized by a very large ovoid blepharoplast. Dividing forms are

never seen in the blood. The common site of multiplication is in the cells of the voluntary muscles and heart and also in the cells of the central nervous system, adrenals, thyroid and bone marrow. In these tissues the flagellate take on a rounded form resembling *Leishmania* and undergoes binary division. Continued division converts the infected cell into a cyst. These division forms assume a trypanosome type and breaking out of the cyst-like cell into the surrounding tissue eventually reach the blood stream. It is this process going on in various important structures that accounts for the extreme variation in symptomatology and pathology.

Chagas has stated that the gametes for the cycle in *Triatoma* arise from parasites developing in the lungs of the vertebrate host. Flagellated parasites enter the lungs, lose the flagellum and become oval in shape, later on dividing into 8 parts. These assume an elongated form and are supposed to enter the red cells of the host. The forms taken up by *Triatoma* multiply in the intestine and then pass to the salivary glands after about eight days. The bug is then infectious when it bites. The consensus of opinion at present time seems to indicate that the cycle in the lung, described by Chagas, belongs to another organism, possibly *Pneumocystis*.

The finding of trypanosomes within the red cells has not been verified by other observers, nor their presence in the salivary glands.

Brumpt does not believe that biting per se is the most important mode of transmission. The adult forms taken up by the bug undergo certain changes in the alimentary tract and multiply. In 8–10 days metacyclic trypanosomes are found in the large intestine. These forms escape in large numbers through the faeces and by scratching or rubbing this material into the bite wound or other abrasions or by direct transference by means of fingers to mucous membrane of mouth or eye infection takes place.

Chagas first placed this trypanosome in the genus *Trypanosoma* but later created the new genus *Schizotrypanum* on account of believing that the intracellular mode of development took place by schizogony. Wenyon and others, however, prefer to retain the organism in the genus *Trypanosoma*, as it is now known that the multiplication occurring within cells is by binary fission and not by schizogony.

Leishmania

Leishmania are found in kala-azar; and in cutaneous lesions in the Orient and in South America. Shattuck, Little, and Coughlin described three cases of granuloma pudendi in every one of which typical *Leishmania* were found in scrapings.

All these *Leishmania* are morphologically alike, although it has been assumed that the generalized infection in man is caused by a distinct species, named the *L. donovani*, which may also produce cutaneous lesions in animals, and sometimes in man.

Leishmania found in cutaneous lesions are said to be of another species, named *L. tropica*, but these may produce generalized infections in animals.

Because kala-azar in the Mediterranean and Caspian areas is associated with a similar disease in dogs while in India the disease has not been found in dogs, it was postulated that still another species of *Leishmania* was responsible in the former localities, and named *L. infantum*.

The cutaneous lesions in South America being more severe and more chronic than are those in the Old World, a fourth species was assumed to be responsible, and named the *L. braziliensis*.

There is no definite evidence for or against the view that all *Leishmania* belong to the same species.

While serological tests are of doubtful value in separating true species, Noguchi tested the agglutinating power of sera from inoculated rabbits on cultures of *Leishmania*. The sera of rabbits inoculated with *L. donovani* agglutinated, in dilution 1–10 and even 1–100, this organism and also *L. infantum*, but no others. Serum from animals inoculated with *L. tropica* agglutinated this organism alone, and the same was true of the serum from the animal inoculated with *L. braziliensis*.

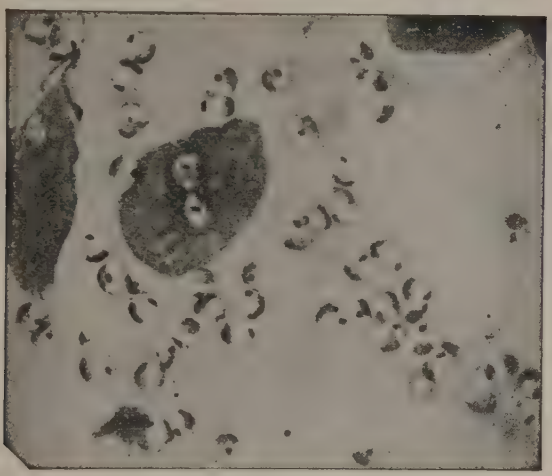


FIG. 99.—*Leishmania tropica*. Smear from granulation tissue of Delhi boil or oriental sore. (*MacNeal from Doflein after J. H. Wright.*)

With animal inoculations we formerly thought that the parasite of kala-azar could be differentiated from that of infantile leishmaniasis by the fact that dogs could not be infected with *L. donovani*, while they were susceptible to infections with *L. infantum*. Donovan and Patton have successfully inoculated dogs with kala-azar splenic material. Patton found the parasites in the liver, spleen and lymphatic glands as well as bone marrow of the inoculated dogs. Consequently we cannot separate the two visceral leishmaniases from a standpoint of susceptibility of the dog. Monkeys are susceptible to both diseases.

As regards separating oriental sore from the visceral leishmaniases Gonder has shown that white mice may be infected with both kala-azar and oriental sore, there being produced in each case a general infection with the presence of parasites in spleen and liver. A point of difference, however, is that the oriental sore mice develop lesions on feet, tail and head which was not observed with the kala-azar

mice. There are some reasons for thinking that in human cutaneous leishmaniasis a generalized infection may precede the local manifestations.

A very interesting point is that the dogs in India never show a natural infection with *L. donovani* while in the regions where leishmaniasis occurs in children the natural infection of dogs is not uncommon, indeed many think the dog the reservoir of virus for the *Leishmania*. It has been suggested that the dogs of India, where kala-azar prevails, may be immune.

Transmission.—Some consider the non-flagellated bodies, which are usually found packed in endothelial cells of spleen, liver, lymphatic glands and bone marrow, as resting stages, the flagellate existence occurring in some other host than its vertebrate one.

Transmission may occur in the case of cutaneous lesions by direct contact. *Leishmania* may also be transferred from such lesions mechanically by any insect. Any blood-sucking insect when feeding on a kala-azar patient may ingest *Leishmania* and if feeding soon again on a healthy individual may infect him. The *Leishmania* may develop in the insect into the leptomonas form, as they might do in a culture tube, and as they do in the bedbug; and yet such insects are not hosts and do not play an important role in transmission of leishmaniasis.

Shortt and Swaminath, also Nicolle and Anderson, record elaborate, prolonged, and convincing experiments showing that bed-bugs (*Cimex*) are not vectors of kala-azar.

Donovan is disposed to incriminate *Triatoma rubrofasciata* as the transmitting agent and furthermore he feels that there has not been sufficient investigation of mosquitoes along this line.

In the female, *Phlebotomus argentipes*, after feeding on kala-azar patients, a heavy leptomonas infection of intestine and pharynx has been found. The distribution of *Phlebotomus argentipes* in India corresponds to the distribution of kala-azar.

In Brazil there exists some evidence that the cutaneous leishmaniasis found there may be transmitted by species of the tabanid family.

Sergent, Adler, and others inoculated material from *Phlebotomus papatasii* into human skin and from the typically developing skin lesion isolated *Leishmania*. At present *Phlebotomus papatasii* is considered to be the probable transmitter of *L. tropica*.

As the dog has been regarded by some as the reservoir of the virus so naturally the transmission of the disease from dog to child through the flea has been considered. Wenyon, tried to infect two young dogs with great numbers of fleas which had previously fed on dogs infected with canine leishmaniasis and at autopsy, five or six weeks later, was unable to find parasites in smears from spleen, liver or bone marrow and did not succeed in obtaining cultures from this material inoculated into tubes of N. N. N. medium.

Common Flagellates of Arthropods.—It must be understood that there is always a suspicion that the flagellate forms noted in arthropod

experiments may be those of nonpathogenic herpetomonad or crithidial species as such forms are common in arthropods and are difficult to distinguish from the flagellate stage of *Leishmania*. For example, *Herpetomonas muscae-domesticae* is common in the intestine of the house-fly. See discussion under Trypanosomidae family for differences, page 404.

Laboratory diagnosis depends on finding the parasite in smears from cutaneous lesions, spleen, blood or culture. Serological and sensitization tests may also aid in establishing a diagnosis of leishmaniasis.

Methods of Obtaining Material. 1. *From cutaneous lesions.*—In ulcerating variety scrapings are unsatisfactory. Carefully sterilize skin at edge of ulcer (iodine). This is essential if the material is to be cultured, since bacteria seem to act unfavorably on *Leishmania*. (This is also indicated by recovery of patients from visceral leishmaniasis after certain bacterial infections.) Make puncture with needle or knife and draw off material into sterile pipette, from which blow the material into the liquid at bottom of a tube of N. N. N. medium of pH7. Keep at 22°–25°C. In 3 days to 3 weeks, according to number of organisms introduced, the flagellates can be found in the tube. Subcultures are easily obtained. A strain of *L. donovani* isolated in 1910 was reported by Nicolle (1925) to be still growing (subcultured 395 times). Material obtained by splenic puncture, or even blood taken from the finger, with care to avoid bacterial contamination, can be used to establish a diagnosis in kala-azar by culturing as described above. Cultures can also be obtained from centrifuged deposit from urine of kala-azar cases; and from material obtained by liver puncture. When few organisms are present they may be missed in smears, but will develop in cultures. A negative culture does not, of course, rule out leishmaniasis.

The oval parasites develop into leptomanad flagellates, from 20 to 22 microns long, by 3.5 microns broad, with a 20 micron flagellum which originates from the blunt anterior end of the body near the blepharoplast.

Parrot and Lestoquard record a technique for bringing out histologic structure of flagellates:

(*a*) Fix 10 min. in Schaudinn's alcoholic sublimate.
(*b*) Transfer to iodine-alcohol.
(*c*) Remove iodine with alcohol, 95%, and dry.
(*d*) Immerse in horse serum 10 min., and dry.
(*e*) Stain with Giemsa.

2. *From spleen by puncture* (Manson and Bahr).—The finding of *Leishmania* in the blood, which should be examined first, will obviate splenic puncture and will also exclude leucocythemia. In doing splenic puncture there is danger of fatal haemorrhage. Statistics indicate 1% mortality after spleen puncture, yet Bousfield did not have a single fatality in 120 spleen punctures.

When liver is enlarged, it being less vascular, and less easily torn, is to be preferred even though the organisms are not so abundant in this organ. Limit movement of diaphragm by firm abdominal binder, to lessen risk of tearing the punctured

organ. While puncture is being made caution patient not to start, and not to breathe.

Since a trace of water in the needle will distort or burst the parasite and render it unrecognizable, the hypodermic needle must be dry. The needle should be connected with barrel of syringe by a short piece of rubber tubing. Little or no blood should be drawn, in order that there may be a greater number of spleen cells, which are the ones required for finding the parasite.

Blow contents of needle out on a slide, spread the film, and after it has dried, stain by Leishman's or Giemsa's method. Many films may have to be examined before a single parasite is seen.

Smears.—In smears of the peripheral blood the parasites if present will be found in polymorphonuclear, or large mononuclear leukocytes. Rarely will there be more than one parasite-containing leukocyte to a slide.

In smears from spleen, the parasite will be in the spleen cells.

The *Leishmania* so found are small, oval, cockle-shell-shaped bodies, measuring 2.5 × 3.0 microns. The protoplasm stains a faint blue and contains a rather large nucleus which is peripherally placed and gives the appearance of the hinge of the cockle-shell. Besides the nucleus we have a second chromatin-staining body which is often rod-shaped and set at a tangent to the nucleus. It consists of the kinetoplast (part of which is a blepharoplast) and axoneme and stains a more intense reddish than the rather more faintly stained pinkish nucleus. There is no flagellum, but the axoneme can often be detected extending from the kinetoplast to the surface of the body, see p. 404 for relationships. One or more vacuoles are common in the cytoplasm. Yeasts often stain in a manner closely resembling *Leishmania* and may exhibit a deeply staining granule. But reproduction is by budding and evidence of this can usually be found.

Serological Tests. *Formol-gel test* or *aldehyde reaction.*—Add a drop of commercial formalin to 1 cc. of serum of a case of kala-azar. The serum will solidify in a few minutes, and very quickly become opaque, like the coagulated white of an egg. This reaction is quite constant in kala-azar disappearing progressively during treatment with tartar emetic and occurs only partially in tuberculosis, leprosy and heavy malarial infections.

Globulin precipitation test.—Mix one part of serum of a case of kala-azar with two parts of distilled water. An opacity is produced. Or pour water on the surface of the serum, a ring effect will be produced.

Sensitization Tests. (*A*) *Animal* (according to Wagener).—Sensitize rabbits by injections of cultural forms of *Leishmania.* Later inject alkaline extracts of *Leishmania* from cultures into skin. An erythematous papule which reaches its height in 48 hours and persists 3 to 5 days will develop. This reaction may be of diagnostic value.

Antigen can be prepared from *L. donovani* or *L. tropica,* not being specific for either parasite.

(*B*) *Human.* Montenegro reports.—Intradermal injections of alkaline extract of *Leishmania* given to patients with leishmaniasis produce a positive reaction in more than 80% cases. A typically positive reaction confirms a diagnosis of leishmaniasis. It is of interest to note that extract from *L. tropica* provoked a specific reaction in Brazilian cases.

Differential Diagnosis.—The leukaemias can be differentiated easily by the blood picture, an important matter because the spleen of splenomyelogenous leukaemia is very friable and the danger from splenic puncture is far greater in this condition than in kala-azar. Banti's disease with its leukopenia shows a rather similar blood picture and can be differentiated surely only by the finding of Leishman bodies in kala-azar.

Undulant fever, typhoid and the paratyphoids are best differentiated by blood cultures or agglutination tests.

It has been noted that artificial pustulation might assist in diagnosis by giving a multitude of polymorphonuclear leukocytes for examination for phagocytized *Leishmania*.

Cochran has noted the advisability of excising a lymphatic gland and making gland smears to examine for *Leishmania*. Others have reported success with gland puncture as utilized in the glands of trypanosomiasis.

Parasites Resembling Leishmania.—Darling has reported from Panama a protozoon somewhat like *Leishmania* in which the cells of lungs, liver, spleen, and lymphatic glands containing numerous parasites about 3 to 4μ in diameter, slightly oval in outline, and contained a large and small chromatin-staining mass. He has given it the name *Histoplasma capsulatum*. It has been suspected that this parasite may be a yeast-like organism.

Another parasite, which seems closely related to *Leishmania* is *Toxoplasma pyrogenes*. This is an oval parasite (2.5 to 6μ) and has one large chromatin mass. The parasites are generally free when noted in the spleen. It is not found in the blood. Clinically we have a splenomegaly. Three cases have been reported, one from the Sudan. While *Toxoplasma* is a sporozoon it has been thought that this parasite may be a new genus related to *Leishmania*. Wenyon is convinced that the supposed *Toxoplasma* was merely a degenerating or badly-fixed *Leishmania*.

Intestinal Flagellates

These parasites of the intestinal tract are separated according to the number of their flagella.

These flagella can easily be counted in a preparation mounted in Gram's iodine solution. For this purpose I take a clean slide and make a vaseline line across it about 1 inch from the end. A drop of the iodine solution is placed on the slide about ½ inch from the vaselined line and a suitable portion of the faeces to be examined is emulsified in it. The edge of a square cover glass is then applied to the vaselined line and allowed to drop on the preparation. By pressure suitable thicknesses of fluid can be examined. There is an absence of current motion. Better, when accessible, is it to use the dark-field illuminator as in this way the flagella are distinctly brought out. The India-ink method is also applicable. Staining of smears by Giemsa's method, following fixation in methyl alcohol or 5% formalin solution, is more satisfactory for flagellates than for amoebae, which, as before stated, should

be fixed in moist smears and stained by haematoxylin. The method of mounting in iodine solution, however, is the one I always use for encysted amoebae.

The intestinal flagellates are classified according to number of flagella, absence or presence of an undulating membrane and of a blepharoplast. Three of these flagellates are but rarely found in the stools and seem to be of little importance. They probably occur as accidental contamination of the human faeces. They are (1) *Cercomonas*, which has a single nucleus and one flagellum free and a second one which turns backward to be attached to the body and then projects posteriorly as a second free flagellum, (2) *Bodo*, which has a single nucleus, but two anteriorly projecting flagella and (3) *Prowazekia* which has, besides the nucleus, a blepharoplast from which arise two flagella. These flagellates can be cultivated on media used for the cultural amoebae and it is thought by some that they at times show amoeba-like stages.

Some authors question the existence of *Cercomonas*, maintaining that such parasites are deformed *Trichomonas*. Wenyon, however, has described *C. longicauda* from cultures of human faeces. As regards *Bodo*, the species *B. urinarius* is now classified as belonging to the genus *Prowazekia*, so that it is a question whether any of the intestinal flagellates belong to the genus *Bodo*.

Dobell's views.—In the opinion of Dobell there is insufficient evidence for regarding any of the intestinal flagellates as pathogenic. As grounds for this view he cites the facts (1) that there is no good reason for considering intestinal flagellates as more common in cases of diarrhoea than in well persons, (2) that parasites of the alimentary canal which do not attack the tissues of the host, as is the case with intestinal flagellates, are not harmful and (3) no method of treatment has yet been discovered which will remove such an infestation. He notes that intestinal flagellates are adapted to a life in a liquid medium and appear in the stools when the intestinal contents become fluid or semi-fluid. In normal stools encysted flagellates alone are found.

The recognized human intestinal flagellates require only the single host, man, and it is questionable whether the human species are found in other animals.

There is an organism, supposed to belong to the moulds, which may be mistaken for an encysted flagellate. It is called *Blastocystis hominis* and has a large central vacuole with a refractile narrow rim which contains one or more nuclei. When stained by Giemsa's stain the central part is very faintly stained while the rim is deep blue.

Trichomonas confusa (T. intestinalis.)—This is a very common parasite in diarrhoeal stools but as to its pathogenicity there is much doubt. It is pear-shaped and about 9 by 14 microns. There are three flagella projecting anteriorly with another one bordering an undulating membrane and projecting posteriorly. An axostyle passes from the anterior to the posterior end. A cytostome is present near the nucleus. There is also a *T. vaginalis* which is found in vaginal secretion of

acid reaction, disappearing when the reaction becomes alkaline as at the time of menstruation. It is somewhat larger than the intestinal form and is not infrequently found in urine. The encysted forms of *Trichomonas* have not as yet been surely recognized.

Chilomastix mesnili (Tetramitus mesnili.)—This flagellate differs from the preceding one in not having an undulating membrane nor an axostyle. It is 6 by 14 microns in size. The three anteriorly projecting flagella are long and slender. There is a very prominent long slit-like cytostome within which is a flagellum.

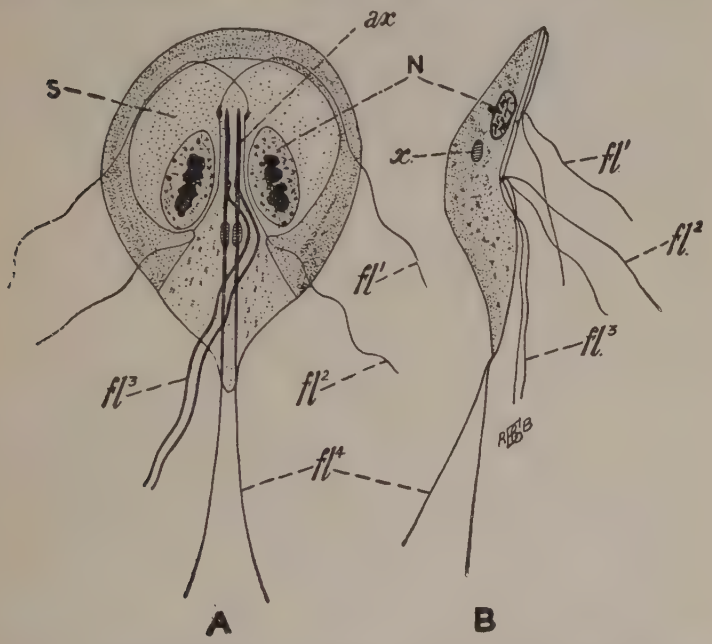


FIG. 100.—*Giardia lamblia*. *A*, Ventral view; *N.*, one of the two nuclei; *ax.*, axostyles; *fl.*¹, *fl.*², *fl.*³, *fl.*⁴, the four pairs of flagella; *s.*, sucker-like depressed area on the ventral surface; *x.*, bodies of unknown function. (*After Wenyon* (277) *from Minchin.*)

The nonflagellate end is very much attenuated. The cysts are about 8μ in length, ovoid with a small projection at the narrower end, and contain one nucleus.

This parasite has been reported not infrequently as a cause of diarrhoeal conditions since its first reporting by Wenyon in 1910.

All the above-mentioned flagellates are found in the large intestines, especially in the region of the caecum.

Giardia lamblia (Lamblia intestinalis.)—These parasites are about 10 × 15μ and have a pear-shaped body with a depression at the blunt anterior end. This depression enables the flagellate to attach itself to the summit of an epithelial cell. Around the depression are three pairs of flagella which are constantly in motion.

Another pair of flagella project from either side of the blunt little tail-like projection. When stained, the parasites have a pyriform shape with two chromatin-staining areas on either side of the anterior end. There are two axostyles present.

In motion they have a slow tumbling sort of progression. These parasites live in the upper portion of the small intestines. The cysts are oval and show the folded flagellate within. Two axostyles and four small nuclei are seen. This infection is frequently associated with a debilitating diarrhoea. Some cases show marked nervous symptoms. In examining the stools of 384 cases, who had practically all been in Gallipoli or Egypt, Woodcock and Penfold, in the King George Hospital, found 98 infected with protozoa as follows: *Giardia*, 22; *Trichomonas*, 14; *Chilomastix* (*Macrostoma*), 11; *E. coli*, 57; *E. histolytica*, 8; *Isospora*, 10.

Embadomonas intestinalis.—This flagellate was reported from Egypt by Wenyon and O'Connor. It is about 6μ long and has a deep depression at the thicker end. Two flagella arise from blepharoplasts on the nuclear membrane of the single nucleus which is near the wide end of the flagellate. The cysts are about 5μ long and pointed at one end.

Infusoria (Ciliata)

The Infusoria are the most highly developed of the Protozoa.

The bodies of Infusoria are oval and may be free or attached to a stalk-like contractile pedicle, as with *Vorticella*, or they may be sessile. The cilia, which are characteristic, may be markedly developed around the cytostome (mouth) and serve the purpose of directing food into the interior, while others act as locomotor organs. The body is enveloped by a cuticle which may have only one opening or slit, to serve as mouth; or it may have a second one, a cytopyge or anus. Usually the faecal matter is ejected through a pore which may be visible only when in use. They usually have a large nucleus and a small one. Infusoria tend to encyst when conditions are unfavorable (as when water dries up in a pond). When the cilia are evenly distributed over the entire body of the ciliates we have the order Holotricha; when ciliated all over, but with more prominent cilia surrounding the peristome, we call the order Heterotricha. It is to this order that the Infusoria of man belong.

Balantidium coli.—This is the only ciliate of importance in man. It is a common parasite of hogs. It is from 60 to 100μ long by 50 to 70μ broad, and has a peristome at its anterior end which becomes narrow as it passes backward. It has an anus. The ectosarc and the endosarc are distinctly marked. The cuticle is longitudinally striated.

These parasites cause an affection similar to dysentery and may bring about a fatal termination. They may produce a pernicious type of anaemia. It is almost impossible to escape noticing the actively moving bodies if a faecal examination is made. When encysted they are round.

McDonald (1922) states that there are two species of the genus *Balantidium* parasitic in the intestinal tract of pigs, namely, *B. coli* and *B. suis* (sp. nov.). He

describes the points of difference between the two and expresses the opinion that *B. suis* does not occur as a parasite of man.

Another ciliate, the *B. minutum*, 25 × 15μ, has also been reported for man.

Nyctotherus faba has a kidney-shaped body and is about 25 by 15μ. It has a large contractile vacuole at the posterior end. It has a large nucleus in the center with a small fusiform micronucleus lying close to it. It has been reported only once for man.

FIG. 101.—*Balantidium coli*. *A*. Fully developed individual, showing the nucleus above at the right and a food particle below. *B* and *C*, Division stages. *D*, Conjugation. (*From Doflein after Leuckart.*)

SPOROZOA

This class of Protozoa gets its name from the method of reproduction—sporulation. These parasites rarely show binary fission. While the sporozoa are found within cells, in the tissues and in internal cavities, as intestine and bile ducts, yet it is as inhabitants of the blood that they have their greatest importance for man—these are known as Haemosporidia. A sporozoon may be either naked and amoeboid or be covered with a distinct cuticle.

NOTE.—Sporozoa are divided into two subclasses—the Telosporidia and the Neosporidia. In the former the vegetative activity of the protozoon goes on to full growth at which time the reproductive activity commences. With the Neosporidia, however, the growth and reproduction go on at the same time.

Among the Telosporidia we have the orders Gregarinida, Coccidiida, and Haemosporidia.

Gregarines are chiefly parasites of arthropods and worms and their presence in man or the higher vertebrates is doubtful.

The subclass Neosporidia is of far less importance in human parasitology, only parasites of the orders Sarcosporidia and Haplosporidia having been reported for man. From an economic standpoint, however, the order Myxosporidia is of great importance—*Nosema bombycis* being the cause of pebrine, a disease destructive to the silkworm.

Coccidiida

The parasites of the order Coccidiida are almost exclusively found in the intestines and in the organs connected with it. In the vegetative stage it lives within an epithelial cell, which it destroys. Afterward it falls into the lumen lined by this epithelial cell and sporulates, either by the method of schizogony or sporogony.

Owing to their egg-like shape, coccidia have often been considered as the ova of intestinal parasites, and *vice versa*. Upon swallowing an oöcyst with its contained sporozoites the membrane of the oöcyst is digested in the duodenum and the sporozoites liberated. They enter epithelial cells, as of intestine, and reproduce by schizogony. After a varying number of nonsexual cycles sporogony commences, sporonts being produced instead of schizonts. The female sporont is fertilized by the microgamete which is an elongated body provided with two flagella. These microgametes are formed from the male sporont and when thrown off from its periphery they enter (usually a single one) the macrogamete. The macrogamete, after fertilization and the formation of a resistant membrane, is termed the oöcyst. Within the oöcyst are found smaller cysts, the sporocysts, in which the sprozoites develop.

FIG. 102.—*Eimeria stiedae.* Oöcyst containing four spores, in each of which two sporozoites are developing. (*From Doflein after Metzner.*)

The cycle is very similar to that of malaria except that no arthropod host is required for the sexual cycle. The spores which are formed in schizogony are known as merozoites.

Merozoites may best be distinguished from sporozoites by the presence of a nuclear karyosome, this being absent in sporozoites. In *Eimeria* we have the oöcyst containing four sporocysts with two sporozoites in each sporocyst while in *Isospora* we have an oöcyst containing two sporocysts with four sporozoites in each.

Eimeria stiedae.—This sporozoon is usually known as the *Coccidium cuniculi* or *C. oviforme*. It is most frequently found in the epithelium of the bile ducts. It has very rarely been reported for man. In these cases (about five) cysts of the liver have been found containing coccidia. The parasite is about 40 × 20μ, and is oval in shape with a double-outlined integument. The sporozoites, which form inside, are falciform in shape. These escape and enter fresh epithelial cells, and thus the process of schizogony goes on. The parasites of the liver are larger than those found in the intestines, these latter being only about 30 × 15μ. In the faeces the form most often found is the oöcyst, about 40 × 20μ. Infection takes place by ingestion of the oöcyst.

E. wenyoni.—Dobell recognizes two other species of *Eimeria* in man, *E. wenyoni* and *E. oxyspora*. There have been about four cases of *E. wenyoni* infection reported. *E. oxyspora* has been reported once. It has a spherical oöcyst (36μ) with 4 sharply pointed sporocysts, thus differing from the oval ones of *E. wenyoni*. When passed in the faeces the contents of the oöcyst are differentiated. Thomson and Robert-

son have shown that these are not human parasites but *E. clupearum* and *E. sardinæ* of herrings which had been ingested and were passing through the human intestine.

Wenyon states that the *Eimeria* oöcysts, found in cases from Gallipoli are round instead of the usual oval. They are about 20 microns in diameter with one or two highly refractile residual bodies. Dobell considers the hepatic coccidiosis of man as due to some species of *Eimeria* which has not been sufficiently studied to justify a name.

Isospora belli.—More than 150 cases of infection with this coccidium have been reported, almost entirely in persons infected in Asia Minor or Egypt. It is doubtful whether it has any effect on the host as cats heavily infected have shown no symptoms. The nonsexual development (schizogony) probably takes place in the epithelial cells of the small gut and the oöcysts pass out in the faeces while still unsegmented, but after a few days show two spores each containing four sporozoites.

The oöcyst, about $28 \times 14\mu$, is pear-shaped. The cyst wall is colorless and inside are two oval spores, $13 \times 8\mu$, with four vermiform sporozoites.

Isospora hominis is a smaller parasite, described by Virchow. The oocysts measure about 15×10 microns.

Haemosporidia

Of the Sporozoa found in the blood (Haemosporidia), the **malarial parasites** are the only ones connected with disease in man.

Malarial Parasites.—There are at least three species of animal parasites which produce human malaria: *Plasmodium vivax*, the cause of benign tertian, *P. malariae* of quartan and *P. falciparum* of aestivo-autumnal. These parasites belong to the haemamoeba type of the order Haemosporidia, of the class Sporozoa and of the phylum Protozoa.

This type of Haemosporidia is characterized by invasion of red cells, amoeboid movement, pigment production and the extrusion of flagellum-like processes from the male sporont after the blood is taken from the animal and allowed to cool.

Other Haemosporidia which are very important in diseases of domesticated animals, but not for man, are those of the **piroplasm type.**

These parasites of the red cells do not produce pigment and do not "exflagellate." It is to parasites of this type that some authorities have ascribed the cause of blackwater fever, a condition undoubtedly connected with malaria.

It has been thought proper by some to consider the malarial parasites as belonging to two genera, the genus *Plasmodium*, characterized by round sexual forms and including *P. vivax* and *P. malariae* and the genus *Laverania*, characterized by crescent-shaped sexual forms and including but one species *L. malariae*, that of aestivo-autumnal malaria.

In addition to man, infections with parasites of a similar nature are found in monkeys (*Plasmodium kochi;* the sexual forms alone seem to be present), in birds (*Haemamoeba relicta;* this organism is usually designated *Proteosoma*). An infection of crows and pigeons of like nature is *Halteridium*. Numerous haemosporidia have been reported for bats, various other mammals, tortoises, lizards, etc.

The full history of the malarial parasite is one of the most interesting chapters in medicine. Laveran discovered the parasite in 1880. In 1885, Golgi noted that sporulation occurred simultaneously with the malarial paroxysm. Koch, Golgi, and Celli demonstrated existence of different species for different types of fever. King and Laveran (1884) considered possibility of mosquito transmission. Manson (1894) formulated hypothesis that gametes were destined to undergo development in the mosquito from observing that flagellated bodies appeared only some time after the blood was withdrawn.

Ross (1895) demonstrated that flagellation takes place in the stomach of the mosquito. McCallum (1897) saw fertilization of macrogametes by microgametes of *Halteridium*. Opie recognized differences in sexual characteristics.

Ross (1898) demonstrated life cycle of the organism of bird malaria (*Proteosoma*), showing formation of zygotes and presence of sporozoites in salivary glands of *Culex* mosquitoes. Grassi and Bignami proved the occurrence in anophelines of a similar cycle for the organism of human malaria. In 1900, infected mosquitoes from Italy were sent to London, where, by biting, they infected two persons (Sambon and Low).

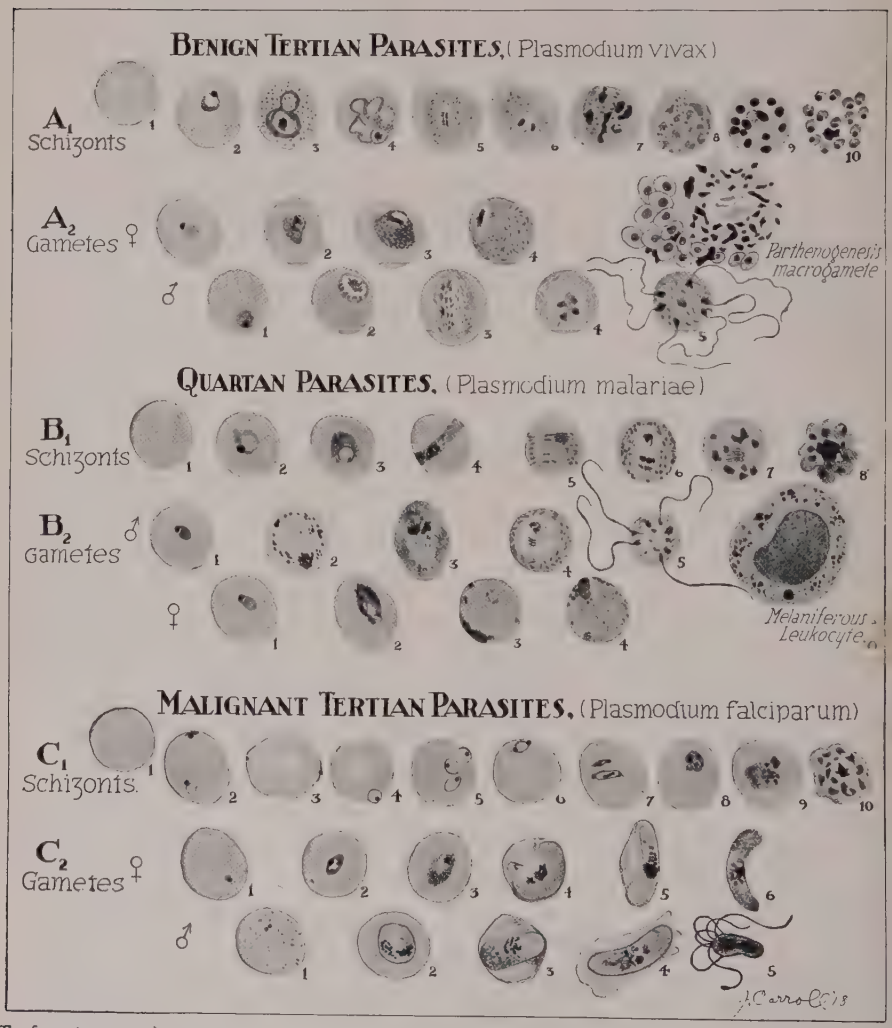
Life History of the Malarial Parasite.—Malaria can be transmitted by subcutaneous or intravenous injection of the blood of a patient with the disease into a well person, the same type being reproduced.

Such a method of transmission is only of scientific interest and the natural method is as follows: An infected anopheline at the time of feeding on human blood introduces through a minute channel in the hypopharynx the infecting sporozoite of the sexual cycle.

When man is first infected by sporozoites we have starting up a nonsexual cycle (schizogony) which is complete in from forty-eight to seventy-two hours, according to the species of the parasite. The falciform sporozoite bores into a red cell, assumes a round shape and continues to enlarge (schizont). Approaching maturity, it shows division into a varying number of spore-like bodies. At this stage the parasite is termed a merocyte. When the merocyte ruptures, these spore-like bodies or merozoites enter a fresh cell and develop as before. At the time that the merocyte ruptures it is supposed that a toxin is given off which causes the malarial paroxysm.

Schizogony.—This nonsexual cycle, termed schizogony, goes on by geometric progression from the first introduction of the sporozoite, but it is usually about two weeks before a sufficient number of merocytes rupture simultaneously to produce sufficient toxin for symptoms (period of incubation).

PLATE OF MALARIAL PARASITES



(*To face page 423*)

DESCRIPTION OF PLATE OF MALARIAL PARASITES

Benign Tertian Parasites

A1. *Schizonts.* 1. Normal red cell. 2. Young ring form. 3. Amoeboid or figure-of-eight form showing Schüffner's dots. 4. Amoeboid form showing increased chromatin (24 to 30 hours). 5. Segmentation of nucleus. 6. Nuclear halves further apart, red cells enlarged and pale. 7. Further division of nucleus. 8. Unusual division form. 9. Typical merocyte. 10. Rupture of merocyte liberating merozoites.

A2. *Female gametes.* 1. Young form showing solid instead of ring-form staining. 2. Half-grown form. 3. Rapidly growing form with compact nucleus and clear vacuolated zone. 4. Full grown macrogametocyte showing eccentrically placed chromatin and much pigment in deep blue stained protoplasm. *Male gametes.* 1. Young form similar to female one. 2. Half-grown form showing central chromatin. 3. Full grown microgametocyte showing large amount of centrally placed chromatin with light blue protoplasm surrounding. 4. Division of chromatin occurring in microgametocyte and developing in wet preparation. NOTE.—Chromatin division in gametocytes does not take place until blood is withdrawn. 5. Spermatozoon-like microgametes developing from the microgametocyte. This only occurs in wet preparations or in the stomach of the mosquito. Parthenogenetic macrogamete. This figure supposed to represent a parthenogenetic female has, since the reporting by Schaudinn, been interpreted as the existence of two parasites in a single red cell.

Quartan Parasites

B1. *Schizonts.* 1. Normal red cell. 2. Young ring form. 3. Older ring form. 4. Narrow equatorial band. 5. Typical band form. 6. Oval form showing division of chromatin. 7. Early stage merocyte. 8. Daisy form merocyte.

B2. *Male gametes.* 1. Young solid form. 2, 3, 4. Developmental stages microgametocytes. 5. Flagellated body in wet preparation showing microgametes developing from microgametocytes. *Female gametes.* 1. Young oval form. 2. Somewhat older stage. 3 and 4. Mature macrogametocytes (same as benign tertian).

Malignant Tertian Parasites

C1. *Schizonts.* 1. Normal red cell. 2, 3, 4, 5, 6. Young ring forms. These are h air-like rings and are the only forms besides crescents to be found in the peripheral blood. *In very heavy infections* or in smears from spleen the following forms are found. 7. Beginning division of chromatin. 8 and 9. Further division. 10. Merocyte.

C2. *Female gametes.* 1. and 2. Young macrogametocytes. 3. Older stage. 4. Development in red cell. 5. and 6. Fully developed female crescents showing clumping of pigment and rich blue color. *Male gametes.* 1. and 2. Developing forms. 3 and 4. Fully developed microgametocytes. 5. Flagellated body developed in wet preparation.

It is considered that there must be several hundred sporulating parasites per cubic millimeter to be capable of producing symptoms. The clinical manifestations are caused by the nonsexual parasites. The sexual parasites are passive in man, and only play a part in the mosquito.

Gametocytes.—After a varying time, whether by reason of necessity for renewal of vigor of the parasite by a respite from sporulation, or whether from a standpoint of survival of the species, sexual forms (gametocytes) develop. Some think that sporozoites of sexual and nonsexual character are injected at the same time. It is usually considered, however, that sexual forms develop from preëxisting nonsexual parasites, the increased reaction of the host being the stimulus which induces the production of sexual forms. It is generally about a week or ten days before we note the appearance

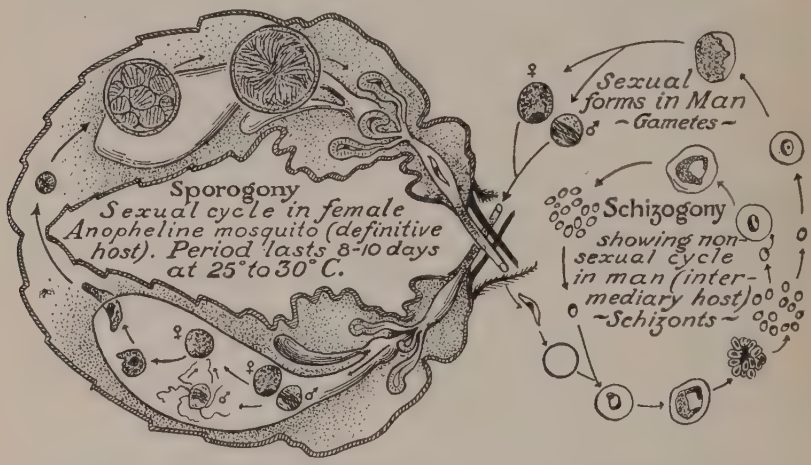

Sporogony
Sexual cycle in female Anopheline mosquito (definitive host). Period lasts 8-10 days at 25° to 30° C.
Sexual forms in Man
~Gametes~
Schizogony
showing non-sexual cycle in man (intermediary host)
~Schizonts~

FIG. 103.—Sexual (sporogony in mosquito) and nonsexual (schizogony in man) cycles of the malarial parasite. The sporogony diagram at the left shows in lower portion the fertilization of the female gamete by the microgamete. The vermiculus stage of the zygote is shown boring into the walls of the mosquito's stomach later to become the more mature zygote packed with sporozoites as shown in the upper diagram of the developmental processes in the mosquito's stomach.

of sexual forms in the blood smears. The developing gametocytes are often termed sporonts. The gametocytes take about twice as long to reach maturity as schizonts. The life of a crescent has been estimated as about ten days and that of the gametocytes of benign tertian and quartan about one-half this period. The gametocytes show two types, the one which contains more pigment, has less chromatin, and stains more deeply blue is the female—a macrogametocyte; the other with more chromatin, less pigment, and staining grayish-green or light-blue is the male— microgametocyte.

Sporogony.—When the gametocytes are taken into the stomach of anophelines, to undergo the sexual phase of their cycle (sporogony), the male cell throws off spermatozoa-like projections, which have an active lashing movement and break off from the now useless cell carrier and are thereafter termed microgametes. These fertilize the

macrogametes, formed from the macrogametocytes by nuclear reduction with formation of polar bodies, and the conjugate body now becomes a *zygote*. This process of exflagellation can be observed in a wet preparation under the microscope. There is first seen a very active movement of the pigment of the male gametocyte and finally long delicate bulbous-tipped flagellum-like processes are thrown off (exflagellated) and push aside the red cells by their progressive motion. MacCallum saw a female *Halteridium* fertilized by the microgamete, after which it was capable of a worm-like motion (vermiculus or oökinete).

By a boring-like movement the vermiculus stage of the zygote goes through the walls of the mosquito's stomach, stopping just under the delicate outer layer of the stomach or mid-gut. In three or four days after fertilization the zygote becomes encapsulated and is then often called an oöcyst. It continues to enlarge until about the end of one week it has grown to be about 50μ in diameter and has become packed with hundreds of delicate falciform bodies. Some contain only a few hundred, others several thousand.

In some of his observations Darling has noted that the zygote of benign tertian malaria grows larger and more rapidly than that of aestivo-autumnal and that its pigment is clumped rather than arranged in belts or lines as with aestivo-autumnal. Darling has also noted that mosquitoes are not likely to become infected unless the gametocyte-carrying man has more than 12 gametocytes to the cubic millimeter of blood.

The capsule of the mature zygote ruptures about the tenth day and the sporozoites are thrown off into the body cavity and distributed throughout the body. Some make their way to the salivary glands and thence, by way of the venenosalivary duct in the hypopharynx, they are introduced into the circulation of the person bitten by the mosquito, and start a nonsexual cycle. As the sexual life takes place in the mosquito, this insect is the definitive host and man is only the intermediate host. The sexual cycle in the mosquito takes about ten to twelve days.

Efficient mosquito hosts.—It must be remembered that only certain species of *Anopheles* are known malaria transmitters; thus Stephens and Christopher, in dissecting 496 mosquitoes of the species *A. rossi*, did not find a single gland infected with sporozoites.

With *A. culicifacies*, however, 12 in 259 showed infection. A mosquito which is capable of carrying out the complete sporogonous cycle is an efficient host and in the case of malaria the mosquito is the definitive host (sexual life of parasite).

Species of Malarial Parasites.—As before stated there are three species of malarial parasites: (1) *Plasmodium vivax*, that of benign tertian—cycle, forty-eight hours; (2) *Plasmodium malariae*, that of quartan—cycle, seventy-two hours; and (3) *Plasmodium falciparum*, that of aestivo-autumnal or malignant tertian—cycle of forty-eight hours.

Plasmodium vivax.—In fresh, unstained preparations, taken at the time of the paroxysm or shortly afterward, the benign tertian schizont, or nonsexual parasite,

is seen as a grayish-white body, round or oval, whose outlines cannot be distinctly differentiated from the infected red cell. They are about one-fifth of the diameter of the red cell and are best picked up by noting their amoeboid activity. In about eighteen hours fine pigment particles appear and make them more distinct. After twenty-four hours the lively motion of the pigment and the projection of pseudopod-like processes, in a pale and swollen red cell, make their recognition very easy. When about thirty to thirty-six hours old the amoeboid movement ceases. Approaching the merocyte stage the pigment tends to clump into one or two pigment masses and one can recognize small, oval, highly refractile bodies within the sporulating parasite.

The gametocytes or sexual forms do not show amoeboid movement, but the fully developed gametocyte, which is generally larger than the red cells, has abundant pigment, which is actively motile in the male gametocyte and nonmotile in the female. The male gametocyte is more refractile, is rarely larger than a red cell and shows yellow-brown, short rod-like particles of pigment. About fifteen minutes after the making of a fresh preparation these male gametocytes throw out four to eight long, slender, lashing processes, which are about 15 to 20 microns long. These spermatozoon-like bodies now break off from the useless parent cell and with a serpent-like motion glide away in search of a female gametocyte, knocking the red cells about in their passage through the blood plasma.

The female gametocyte is larger than a red cell, is rather granular and has more abundant dark-brown pigment than the male.

In dried smears, stained by some Romanowsky method, as that of Wright, Leishman or Giemsa, we note small oval blue rings, about one-fifth of the diameter of the infected yellowish-pink erythrocyte. One side of the ring is distinctly broader than the rather fine opposite end, which seems to hold a round, reddish-brown dot, the chromatin dot, this completing the resemblance to a signet ring. These small tertian rings of the nonsexual parasites (schizont) are seen about the time of the commencement of the sweating stage of the paroxysm. Two chromatin dots in the line of the ring are rare as is also true of more than one ring in a red cell.

When the parasite is about twenty-four hours old we note that it contains much pigment and has an amoeboid or multiple figure-of-eight contour, is about three-fourths the size of a red cell and that the infected red cell is about one and one-half times as large as in the beginning and presents a washed-out appearance. It is an anaemic-looking cell. We also note, as characteristic of a benign tertian infection, reddish-yellow dots in the pale red cell, which are known as Schüffner's dots. These, practically, are peculiar to benign tertian.

A few hours before the completion of its forty-eight hour cycle the contained pigment begins to clump, the chromatin to divide and, finally, we have a sporulating parasite, in which the 16 to 20 small, round, bluish bodies, with chromatin dots, are irregularly distributed over the area of the merocyte.

The gametocytes, or sexual parasites, show a thicker blue ring and have the chromatin dot in the center of the ring. The pigmentation of the half-grown gametocytes is more marked than that of schizonts of equal size. The shape of the gametocytes is not amoeboid, as is that of the twenty-four- to thirty-six-hour-old schizont, but round or oval. *The full-grown gametocytes have the pigment distributed and the chromatin in a single aggregation—just the opposite of nonsexual parasites*

The male gametocyte stains a light grayish-blue and has a very large amount of chromatin, usually centrally placed. The female gametocyte stains a pure blue, has only about one-tenth as much chromatin as plasma, with the chromatin often placed at one side. The pigment of the female gametocyte is dark-brown while that of the male is yellowish-brown.

Plasmodium malariae.—In fresh preparations the young quartan schizont has only slight amoeboid movement and, as development proceeds, the rather dark-brown, coarse pigment tends to arrange itself peripherally about the band-shaped or oval parasite.

The infected red cell shows but little change. At the end of seventy-two hours the rather regular daisy-form of merocyte is more distinct than that of the benign tertian merocyte.

The distinctions between the male and female gametocytes are similar to those of the benign tertian gametocytes. In Romanowsky-stained smears it is difficult to

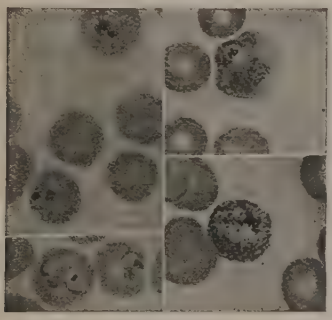


FIG. 104.—Tertian malarial parasites, one red cell showing malarial stippling. (*Todd.*)

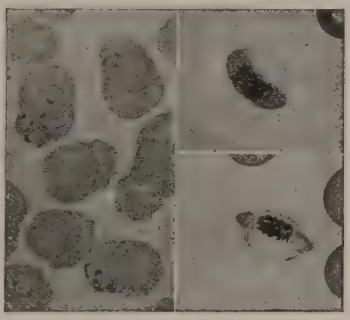


FIG. 105.—Aestivo-autumnal malarial parasites, and small ring forms and crescents. (*Todd.*)

distinguish the young quartan schizont from the benign tertian one but, after twenty-four hours, the tendency of the quartan schizont to assume equatorial band-forms across a red cell of normal size, staining characteristics and absence of Schüffner's dots make the differentiation easy. In the fully developed sporulating parasite or merocyte the eight merozoites assume a regular distribution giving it the appearance of a daisy.

The gametocytes show practically the characteristics of the benign tertian ones but are smaller.

Plasmodium falciparum.—The young schizont of malignant tertian is extremely difficult to detect in fresh preparations, there being noted early in the rather long continued, hot stage, only small crater-like dots, about one-sixth of the diameter of a red cell, which, however, show an active amoeboid movement.

Later on in the hot stage these ring-like dots enlarge to become about one-third of the diameter of a red cell, most often occupying the periphery of the infected red cell. About this time, or at the very commencement of the pigmentation, the

schizont-containing red cells disappear from the peripheral circulation so that the further development is rarely observed in blood specimens.

The infected cell is brassy in color and shrunken in shape—it shows evidences of degeneration. The gametocytes appear as crescent-shaped bodies, which are absolutely characteristic of malignant tertian, the male gametocyte being more hyaline and delicate while the female is more granular and larger.

In Romanowsky-stained preparations we see, while the fever is sustained, small hair-like rings, with geometrical outline, with frequently two chromatin dots in one end of the ring and a single cell often showing two or more of these young rings. The rings are often seen as if plastered on the periphery of the red cells or as if having destroyed a rounded section of the rim of the red cell. As the fever declines the rings tend to disappear from the peripheral circulation. The infected red cells often show polychromatophilia and distortion, but may be normal in appearance.

Unstained Specimen (Fresh Blood)

	P. vivax (benign tertian)	P. malariae (quartan)	P. falciparum (malignant tertian) (aestivo-autumnal)
Character of the infected red cell.	Swollen and light in color after eighteen hours.	About the size and color of a normal red cell.	Tendency to distortion of red cell rather than crenation. May appear normal. (Brassy color.)
Character of young schizont.	Indistinct amoeboid outline. Hyaline. Rarely more than one in r.c. Active amoeboid movement. One-third diam. of r.c.	Distinct frosted-glass disc. Very slight amoeboid motion.	Small, distinctly round, crater-like dots not more than one-sixth diameter of red cell. Two to four parasites in one red cell common. Shows amoeboid movement until appearance of pigment.
Character of mature schizont.	Amoeboid outline. No amoeboid movement.	Rather oval in shape. Sluggish movement of peripherally placed coarse black pigment.	Only seen in overwhelming infections. Have scanty fine black pigment clumped together.
Pigment.	Fine yellow-brown, rod-like granules which show active motion in one-half-grown schizont. Motion ceases in full-grown schizont.	Coarse almost black granules. Shows movement only in young to half-grown schizont.	Pigmented schizonts very rare in peripheral circulation except in overwhelming infections. Tends to clump as eccentric pigment masses almost black in color.

In old aestivo-autumnal cases, or those with severe infection, we may see adult rings and merocytes, which latter are smaller than those of benign tertian and show from 10 to 12 irregularly placed merozoites and a sharply clumped mass of pigment.

The gametocytes are the striking crescent-shaped bodies and these show the distinctions of blue staining for the female, with lighter gray-green staining and abun-

STAINED SPECIMEN

	P. vivax (benign tertian)	P. malariae (quartan)	P. falciparum (malignant tertian) (aestivo-autumnal)
Character of infected red cell.	Larger and lighter pink than normal red cell. Shows "Schüffner's dots."	About normal size and staining.	Shows distortion and some polychromatophilia and stippling. Rarely we have coarse cleft-like reddish dots—Maurer's spots.
Character of young schizont.	Chromatin mass usually single and situated in line with the ring of the irregularly outlined blue parasite.	Rather thick round rings which soon tend to show as equatorial bands.	Very small sharp hair-like rings, with a chromatin mass protuding from the ring. Often appears on periphery of red cell as a curved blue line with prominent chromatin dot. Frequently two chromatin dots.
Character of half-grown schizont.	Vacuolated or Fig. 8 loop-like body with single chromatin aggregation. Schüffner's dots.	More marked band forms stretching across r.b.c.	Not often found in peripheral circulation. Chromatin still compact.
Character of mature schizont.	Fine pigment rather evenly distributed in irregularly outlined parasite.	Coarse pigment rather peripherally arranged in an oval parasite.	Very rarely seen in peripheral circulation in ordinary infection. Pigment clumps early.
Character of merocyte.	Irregular division into 15 or more spore-like chromatin dot segments. Mulberry.	Rather regular division into eight or ten merozoites. Daisy.	Sporulation occurs in spleen, brain, etc. Rarely in peripheral circulation. Eight to ten chromatin-staining merozoites. (In culture, 32.)
Character of macrogametocyte.	Round deep blue. Abundant, rather coarse pigment, chromatin at periphery.	Round, similar to P. vivax but smaller.	Crescentic, deep blue; pigment clumped at center; chromatin scanty and in center.
Character of microgametocyte.	Round, light greenish-blue; pigment less abundant; chromatin abundant and located centrally or in a band.	Round like P. vivax.	More sausage-shaped than crescent. Light-blue. Pigment scattered throughout. Chromatin scattered and in greater quantity but difficult to stain.

dance of chromatin for the male. The chromatin-staining of crescents does not stand out so well as that of the round form gametocytes of benign tertian and quartan.

As regards differentiation of species and cycle the examination of stained smears is more satisfactory and definite, as well as less time-consuming.

Still, one obtains many points of differentiation in the fresh preparation and should study such a preparation while carrying out the staining of his dried smear.

Blood platelets are the objects most frequently mistaken for malarial parasites in stained blood, and the vacuoles in fresh blood.

Central vacuolation of red cells is common in malarial anaemia and may be mistaken for nonpigmented parasites.

Malarial rings are usually peripheral and do not vary in size as one focuses up and down as do the central vacuoles.

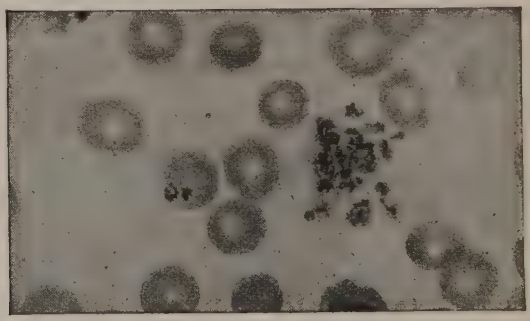


FIG. 106.—A cluster of blood-platelets and two platelets lying upon a red cell and simulating malarial parasites (×1000). (*Todd.*)

Cultivation.—See page 52.

Certain Questions Connected with the Life History of the Malarial Parasite in Man. (1) *Multiple infections.*—Variations in paroxysms may be produced by infected mosquitoes biting on successive nights, so that one crop will mature and sporulate twenty-four hours before the second. This would give a quotidian type of fever. In aestivo-autumnal infection anticipation and retardation in the sporulation cause a very protracted paroxysm, lasting eighteen to thirty-six hours; this tends to give a continued or remittent fever instead of the characteristic intermittent type.

2. *Mixed infections.*—When two species of parasites are present in the same case we have a mixed infection. Mixed infections of malignant tertian and benign tertian are the most common, next those of quartan and malignant tertian and very rarely those showing quartan and benign tertian. All three species have been found in a single individual.

3. *Malarial index.*—Mosquito dissection is one method of determining the endemicity of malaria or the malarial index. There are two other methods: (1) By noting the prevalence of enlarged spleens, and (2) by determining the number of inhabitants showing malarial parasites in the blood.

A spleen index of 50 would mean that 50% of the people had enlarged spleens. Bass has employed the history of a malarial attack during the preceding year as

a factor in determining the percentage of incidence. Thus of 31,459 persons included in a malarial survey of Bolivar County, Miss., 40.3% gave a positive history of malaria and showed a parasite incidence of 28.96%. There was a negative history of malaria during the preceding year in 59.70%, yet of these 15.93% showed parasites. Thick-film methods were used for demonstrating parasites.

The index is best determined from children between two and ten years of age, as children under two show for a general average too high a proportion of parasites in the peripheral blood while those over ten years of age show too great an incidence of enlarged spleens. Barber working in the Philippines with children from five to ten years of age obtained a spleen index of 13.3 and a parasitic index of 11.

4. *Congenital malaria.*—There has been some question as to the possibility of congenital malaria. Heiser has reported the case of an infant which showed crescents in its blood by the end of one week from birth. The mother showed the same infection and the child must have been infected through the placental circulation.

Clark in numerous examinations of the blood of the newborn failed to find infection even when the mother's blood teemed with parasites. In one case where the child showed infection shortly after birth there had been an accident to the placenta and he believes that instances of so-called congenital malaria are to be explained in this way.

5. *Immunity.*—There is no real immunity to malaria. When absence of acute symptoms seems to indicate the existence of immunity, there is present a continuation of an infection, not recognized because the parasites are not in sufficient numbers to give rise to fever but shown by the development of fever if the patient becomes chilled or fatigued or otherwise depressed.

This apparent immunity is also kept up by reinfection, because if natives leave the locality for a length of time they lose it. Patients who show this apparent immunity to one form of malaria have no such resistance to the other types. Bass states that immune bodies are produced in malaria and that immune processes contribute to control of the infection, but that immunity is not lasting and is not effective against new infection.

6. *Relapses.*—Relapses are distinct features of malarial diseases, the tendency being most marked in quartan and least so in malignant tertian. A relapse after an interval of two years is very rare in malignant tertian but periods as long as nine years may intervene between attacks of quartan fever.

Relapses are intimately associated with conditions which tend to lower the body resistance, so that exposure to cold or wet or hot sun may bring on an attack. Alcoholic or venereal excesses, as well as errors of diet, may be provocative. Persons returning home from the tropics often experience relapses as they approach the cooler climate of the temperate zone. It has been well stated that the old resident of the tropics owes his condition of health rather to education than acclimatization—experience has taught him discretion.

There are several explanations of relapses of which the one supported by Ross and Bignami seems most reasonable. It is that the disappearance of nonsexual parasites is only apparent and that they continue their cycle but in insufficient numbers to excite symptoms. Recently, however, the question of the presence of specialized nonsexual resistant forms has arisen. S. P. James suggests that these hitherto, because

of their general resemblance to gametocytes, unrecognized forms may be the cause of true relapses.

7. *Latent malaria.*—The persistence of a malarial infection, in the absence of clinical and to a great extent of laboratory manifestations, is shown by the occurrence of relapses, so that the section treating of malarial relapses applies equally to this paragraph. In addition to the factors influencing relapses, such as exposure to sun, rain and excesses of various kinds, we note a particular tendency for a latent malaria to develop activity following surgical operations and childbirth. In untreated latent cases we may have delayed healing of surgical operations.

8. *Types of pernicious malaria.*—It is customary to divide pernicious malaria into the following divisions—(1) Cerebral, (2) Algid, (3) Bilious Remittent and possibly, also (4) Pneumonic and (5) Cardiac types.

We do not understand why in one case sporulating parasites should plug the capillaries of the central nervous system, with the production of conditions resembling well recognized nervous diseases, while in another case the damage is done the intestinal mucosa, pancreas or lungs. At any rate these pernicious manifestations of malaria should always be kept in mind when a case of sudden cerebral involvement or acute abdominal disease shows itself in a patient in a malarious country and a blood examination should be promptly made.

As explanations of perniciousness are given: (1) The very large number of red cells infected and destroyed by the malarial parasites; (2) the throwing off at the time of sporulation of the merocyte of a large amount of toxic material owing to the presence of such a large number of disintegrating merocytes, and (3) from the plugging of the capillaries of important internal organs by adult parasites. This may arise as the result of (*a*) the sporulating parasites acting as emboli, being too large to pass the lumen of the capillary; (*b*) from degenerative changes or distention with pigment of the endothelial cells lining the capillaries, or (*c*) as the result of an ovoid shape on the part of the malignant tertian parasite there is an inability to pass through capillaries which the flattened benign parasites can do by infolding (Bass), or (*d*) resulting from the tendency of malignant tertian parasites to agglutinate.

The Thompsons note 32 merozoites as maximum number in sporulation of *P. falciparum* while *P. vivax* has usually 16 or more, but never as many as 32. This would explain the shorter incubation period of malignant tertian.

9. *Malarial toxin.*—Nature of the toxic material thrown off by the parasite at the time of sporulation: Rosenau's experiments tend to show that there is a fever-producing toxin thrown off at this time. Other authors have thought that an haemolysin and an endotheliolysin were thrown off at the same time. Brown considers that the pigment produced by the parasite, in its metabolism of the haemoglobin of the red cell, may act as an haemolysin, he having found that intravenous injections of haematin were capable of producing marked anaemia. It is well known that a far greater number of red cells are destroyed in a paroxysm than would be accounted for by the actual percentage of red cells destroyed by parasites. The endothelial cells take up actively this malarial pigment or haemozoin and are damaged or destroyed thereby. Haematin injections also tend to destroy leukocytes and platelets.

Rowley-Lawson is of the opinion that the greater red cell destruction than would be represented by percentage of cells showing parasites is explained by parasites

migrating from cell to cell so that many red cells may be destroyed by a single parasite.

10. *Anaphylaxis and the paroxysm.*—Abrami has brought forward evidence in favor of the malarial paroxysm being due to the outpouring of merozoites into the blood plasma which act as foreign antigen. It is noted that the dissemination of merozoites takes place some hours before the cold stage which is one of the manifestations of anaphylactic shock. A leukopenia and lowering of the blood pressure preceding the paroxysm are considered evidence of a haemoclastic crisis.

11. *Extracellular location.*—It is usual to consider the parasite as developing within a red cell and in this position to destroy the red cell. Rowley-Lawson, however, thinks that the parasites are exclusively extracellular and that they adhere to the red cell by loop-like pseudopodia which encircle a portion of the red cell and digest the haemoglobin of such an area.

12. *Transmission to larvae.*—There has been an idea that sporozoites might enter the ovaries and ova as well as the salivary glands so that a second generation of mosquitoes might transmit malaria. There is no proof that such a method is ever operative.

13. *Effect of quinine on malarial parasites.*—Bass thinks that quinine makes the red cell permeable to the lytic action of serum. Quinine not only causes parasites to disappear from the peripheral circulation but produces degenerative changes in such parasites as may remain. Such parasites lack definiteness of outline and show poor chromatin staining (quinine-affected).

Mayne and Wenyon have shown that quinine is much more potent against the schizonts of malignant tertian and the gametocytes of tertian malaria than vice versa. The tertian gametocytes are emasculated by the action of quinine and hence incapable of exflagellation. Muehlens and Kirschbaum have by actual experiment shown that the gametocytes from a case of malignant tertian malaria under the influence of quinine will undergo sporogony in the insect host and produce infective sporozoites.

14. *Provocative measures.*—Fatigue and refrigeration are well recognized means of inducing a relapse in malaria and hence may be of value as diagnostic measures. Other provocative agents are quinine administered in small doses over ten days or two weeks and subcutaneous injections of epinephrin (the best procedure) or antityphoid vaccine. Sunlight is a potent agent in the production of relapses, its effect being probably due not so much to the vital depression possibly induced by undue exposure but rather to the action of the shorter light waves.

Piroplasms

Belonging like the malarial parasite to the Haemosporidia we have a group of parasites known as the PIROPLASMS. Some consider *Babesia* the correct name for these parasites but they are better known under the name *Piroplasma*. They are minute organisms, usually pear or oval shaped, which invade the red corpuscles. They produce no pigment but destroy the corpuscles and set free the Hb. which is excreted in enormous amounts by the kidneys. It is this which gives the name red-water to the disease usually designated Texas fever of cattle.

The cause of this disease is *B. bovis* (*B. bigemina*) and the parasite is transmitted by a tick, *Margaropus annulatus*. There is also a disease of dogs called malignant jaundice of dogs which is caused by *B. canis* and also transmitted by a tick. A disease of sheep, known as "Carceag," caused by *B. ovis* and transmitted by *Rhipicephalus bursa*, exists in the Balkan regions. Organisms of this kind have been thought of in connection with blackwater fever of man. Seidelin has claimed that a parasite of similar nature, *Paraplasma flavigenum*, was the cause of yellow fever.

At one time spotted fever of the Rocky Mountains was supposed to be due to a parasite named *Babesia hominis*. In Africa there is a disease of cattle, called "Rhodesian fever," which is caused by a rod-shaped parasite in the red cells. (*Theileria parva*.) The genus *Theileria* is characterized by bacilliform or coccoid shape rather than the pear shape of *Babesia*.

Bartonella bacilliformis.—This parasite which is now regarded as a *Bacterium* was at one time thought to be a piroplasm. The organism was noted by Barton in 1905 and studied by Strong in 1914. The parasites are usually in the form of minute rods about 2 microns long lying in red cells. They are motile and stain readily. Strong connected the parasite with Oroya fever alone, while Noguchi gives it as the cause of both Oroya fever and verruga peruviana.

FIG. 107.—Miescher's sac from the musculature of a hog. × 30 diameters. (*After Ostertag.*)

Sarcosporidia

Sarcosporidia are sporozoa found in the striped muscles of various mammals and birds. The present view is that we have only one genus of these parasites, viz., *Sarcocystis*. Life history is unknown. They are common in the pig and mouse and have been reported for man in three well authenticated cases. In the last one reported, Darling found these protozoa in the biceps muscle of a negro patient in Panama. In Baraban's case the laryngeal muscles at autopsy were found to show cysts about $\frac{1}{15}$ inch long which contained sickle-shaped sporozoites about 9μ long.

They are known also as Miescher's tubes when in muscle fibers. These Miescher tubes or sarcocysts are elongated, tubular bodies which distend the muscle fibers. They vary greatly in length and thickness. The sarcocyst of *Sarcocystis tenella* may reach a length of 16 millimeters. These sarcocysts are filled with sickle- or oval-shaped spores, from 7 to 15μ long by 3 or 4μ wide. These spores (Rainey's corpuscles) lie in masses which fill the numerous chambers into which the sarcocyst is divided by partition-like walls. There is an enveloping capsule for the sarcocyst which may

show striations. In some places more than 50% of the sheep and pigs may show infection.

It is never parasitic for invertebrate hosts and while occasionally found in birds and reptiles it is preëminently a parasite of the higher vertebrates. As a rule, it is a harmless parasite but the *Sarcocystis muris* is very pathogenic for the mouse and has been transmitted by feeding mice with the flesh of infected mice.

Haplosporidia

Haplosporidia are chiefly parasites of invertebrates. The human species is called *Rhinosporidium kinealyi* or *R. seeberi*.

Rhinosporidium seeberi.—It causes pedunculated tumors of nasal cavity. When the tumor is incised it is seen to contain many white particles, about 250μ in diameter. These are the cysts which contain the pansporoblasts which are very small at the periphery of the cysts and quite large in the center of the cysts. The ones in the center are the mature ones and from the contained spores, 8 to 16 in number, have a mulberry appearance. When the cyst is mature the pansporoblasts escape through an opening in the cyst wall and thus extend the infection. Most of the cases have been reported from India. The mode of infection is unknown. The systematic position of *Rhinosporidium* is *sub judice* Ashworth classifying them in the lower fungi (*Phycomycetes*).

Chlamydozoa

These organisms are generally considered as being protozoal in nature and as a rule belong to the filterable viruses, which is the designation for the infectious principles of those diseases in which filtration of defibrinated blood or serum through a Berkefeld filter, capable of holding back so small an organism as the *B. melitensis*, does not prevent the infection being transmitted when introduced by the proper atrium of infection. The Chlamydozoa are also characterized by the occurrence of "cell inclusions."

The best known infections of this group of diseases in man are smallpox, vaccinia, rabies, trachoma, molluscum contagiosum, and foot-and-mouth disease. There are many such infections in other animals. The cell inclusions are regarded as products of cellular reaction to a virus which is more or less impossible of demonstration. The discovery of exceedingly minute granules in some of these diseases, as in variola and trachoma, has suggested that, as a reaction to the invasion by such a granule, the cell throws an enveloping mantle about the invading particle. To designate this we use the name Chlamydozoa.

The generic name *Cytorhyctes* has been applied to certain of these viruses; thus *C. vacciniae* develops within the epithelial cells of stratified epithelium. In vaccinia, Councilman and his colleagues consider that the development takes place only in the cytoplasm of the cell. In variola, however, the developmental cycle affects the nucleus.

Cytorhyctes luis, once reported as the cause of syphilis, sporulates in the blood vessels and in the connective tissue, not in epithelial cells.

Cyclasterion scarlatinale was reported by Mallory to have been found in the skin in four cases of scarlet fever.

Proflagellata (Spirochaetacea)

Owing to the prestige of Schaudinn, his opinion that the spirochaetes were to be considered as protozoa and not bacteria was generally accepted. Following the observations of Dobell, Calkins, Wenyon and others the tendency now is again to place these organisms with the bacteria. Until the question of their true position is more definitely settled it has seemed advisable to place the spirochaetes in a group separate from the definitely protozoal organisms and to use the designation Proflagellata, proposed by Doflein for organisms supposed to be transitional from bacteria to flagellates. Stiles groups these organisms of uncertain position in the Spirochaetacea Fantham, 1908 and Bergey in the class Schizomycetes, order Spirochaetales Buchanan, 1918.

Schaudinn regarded the spirochaetes as allied to certain small slender types of trypanosomes and believed he had observed in certain of them such flagellate structures as nucleus and blepharoplast, flagellum and undulating membrane; but later he doubted their trypanosomal structure.

The advocates of the protozoal nature of spirochaetes call attention to arthropod transmission and longitudinal division, but as to the latter the opinion is now almost universal that fission of spirochaetes is transverse as with bacteria.

Medical authorities generally name the organisms of relapsing fever, syphilis, yaws, yellow fever, Weil's disease and rat bite fever as *Spirochaeta*, but it is clear that the organism to which this name was given by Ehrenberg, in 1833, is quite distinct from the spiral organisms of the diseases noted above. *Spirochaeta plicatalis* has a long, highly flexible, central axial filament surrounded by a regularly wound layer of protoplasm, usually of great length, and is not parasitic, but free-living in water. It multiplies by transverse fission and does not have membrane or flagellum. Sambon proposed the name *Spiroschaudinnia* for blood spirochaetes, such as those of relapsing fever. The generic name *Borrelia* however has priority, as noted under Nomenclature, p. 375. Some authors place the blood spirochaetes as well as the tissue ones under *Treponema*, but it would seem well to include in this genus only the distinctly tissue parasites of syphilis and yaws. Noguchi used the name *Spironema* for the blood spirochaetes and noted for them a tendency to straighten out or vary their undulations when in motion or after death, while *Treponema* maintains a fairly constant ridigity of its corkscrew-like spirals. Noguchi notes for *Leptospira* the presence of minute elementary spirals running throughout the body. There is entire absence of flagella as well as of undulating membrane. The caudal portion of the spirochaete is remarkably flexible. While in motion the whole body seems drawn into a straight line except for the hook formation of one or both terminal

portions. Propulsion seems to occur by the rotary motion of the hook and progression is in the direction of the straight end. Should both ends become curved, progression ceases. The organisms of yellow fever and infectious jaundice belong to this genus. These organisms differ from the other blood spirochaetes in that they resist the action of 10% saponin. The genera *Saprospira* and *Cristispira* do not occur in man.

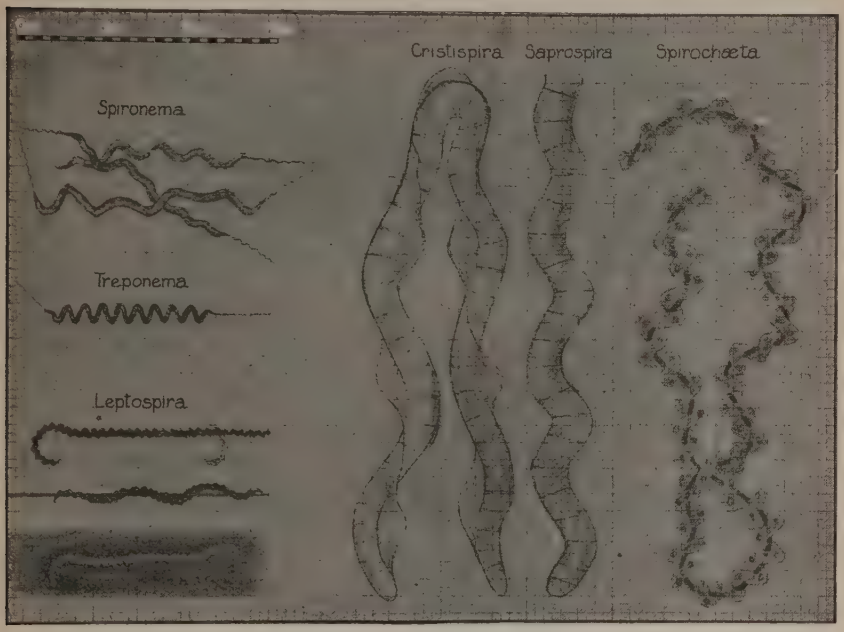


FIG. 108.—Diagram contrasting the characteristic features and relative proportions of *Borrelia* (*Spironema*), *Treponema*, *Cristispira*, *Saprospira*, *Spirochaeta*, and *Leptospira*. The scale in microns is given in the upper left-hand corner of the figure. (*After Noguchi in Journal of Experimental Medicine.*)

Spirochaetacea

1. *Spirochaeta* Ehrenberg, 1833. Large free-living fresh-water and marine forms. Type *S. plicatilis* (500 × 0.75 microns), cylindrical, with regular spirals 1.5 microns apart. Has an elastic flexible axial filament but no crista nor flagella. Not dissolved by bile salts or saponin in 10% solution.

2. *Saprospira* Gross, 1911. Large free-living marine and fresh-water forms; type *S. grandis* (100 × 0.8 microns). Is divided internally into chambers by many transverse septa. Organism disposed in numerous relatively rigid undulating curves. There are no flagella nor is there an undulating membrane (crista).

3. *Cristispira* Gross, 1910. Large spirochaetes parasitic in alimentary tract of oysters and other shell-fish; type *C. balbianii*, Certes, 1882 (45 to 90 × 1.8 microns

with obtuse ends, cylindrical and composed of 2 to 5 large irregular flexures). Has a distinct and flexible longitudinal crest and an internal chambered structure like *Saprospira*.

4. *Borrelia* Swellengrebel, 1907. Type *B. gallinarum*. Various sizes, no axial filament, no crista, nor undulating membrane. All disintegrated by 10% saponin and bile salts; 5 to 14 microns in length by 0.5 micron in width, flexible and snake-like. In man *B. recurrentis*, *B. novyi*, *B. duttoni*, *B. berbera*, *B. carteri*, *B. vincenti*, *B. buccale*, *B. eugyrata*, *B. bronchiale*, etc. Includes *B. anserina* of geese, *B. theileri* and many others.

5. *Treponema* type *T. pallidum* Schaudinn, 1905. Shaped like a corkscrew, pointed ends, 8 to 14 × 0.3 micron with from 6 to 12 turns of the spiral. With dark ground appears as silvery delicate corkscrew in motion. Imperfect illumination may show them as dots. Curves rigid while in *Borrelia* they tend to straighten out. Members of both these genera (German theory) composed of ecto and endoplasm, the former being continued beyond the latter forming the attenuated ends.

6. *Leptospira* Noguchi, 1917, type *L. icterohaemorrhagiae*—Inada and Ido, 1914. 7 to 14 microns in length, 0.3 micron in width, with pointed ends and a spiral amplitude of 0.45 micron; one or more gently undulating curves. Terminal filament, axial filament, and undulating membrane absent. Resist 10% saponin but dissolved by bile salts. Three pathogenic species, *L. icterohaemorrhagiae*, *L. hebdomadis*, and *L. icteroides* (*L. interrogans*). Progress by rotary motion with one end hooked.

The Blood Spirochaetes

Spirochaetes of Relapsing Fevers.—There is a group of tropical fevers more or less identical clinically with European relapsing fever and

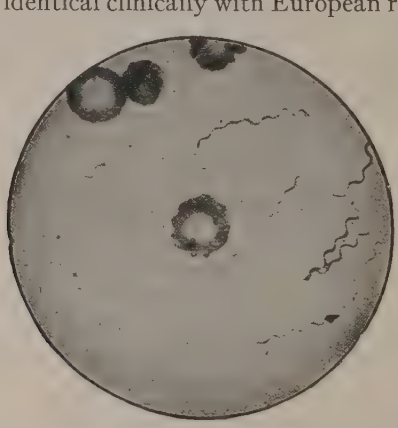


FIG. 109.—*Spirochaeta* of relapsing fever from blood of a man. (*Kolle and Wassermann.*)

caused by spirochaetes closely allied to *Borrelia recurrentis* (*Spirillum obermeieri*). It seems probable that the relapsing fevers of East and

West Africa are caused by a single species, *B. duttoni*, which is transmitted by a tick, *Ornithodoros moubata*, while that of Northern Africa is caused by another species, *B. berbera*, which is transmitted by lice, either *Pediculus vestimenti* or *Pediculus capitis*. Another species of spirochaete, *B. carteri*, is supposed to cause the relapsing fever of India and it seems probable that its transmission is brought about by infected lice.

FIG. 110.—*Borrelia novyi*. (*Todd.*)

Besides the above species of spirochaetes others have been reported, as *B. novyi* for American and *B. persica* for Persian relapsing fever. The view taken by Nuttall, that these various names may be of convenience in the study of relapsing fevers but that there is no adequate morphological difference to justify them as species, seems worthy of acceptance. It has been shown that the separation of these spirochaetes on the basis of susceptibility of laboratory animals and cross immunity reactions is untenable. Agglutination of certain strains by their specific sera, however, is a reliable means of separation.

Borrelia duttoni.—Leishman considers that when spirochaetes are taken into the alimentary tract of the tick there is a breaking up of the spirochaetes into small granules which reach the Malpighian tubules. They also invade the ovary and the ova. It was thought that these granules were the infecting agents and that they were excreted in the fluid of the coxal glands or passed out with the faeces. It has been claimed that these granules have no relation to the infection, which is due to spirochaetes as such. Not only does the tick itself become infected by the ingestion of blood containing spirochaetes but further transmits the infection to its progeny.

It may be stated that spirochaetes as such may be found in the secretion of the coxal glands as well as in the faeces. This coxal fluid dilutes the thick faeces and makes an emulsion which is smeared out by the body of the tick in the area of the bite puncture.

At any rate this infection of man seems to be acquired by the contamination method, the material from faeces and coxal glands being rubbed into the wound made by the tick bite. There may be quite a local reaction at the site of the bite.

Borrelia duttoni has been cultured by Noguchi. See p. 53. In such cultures he has noted longitudinal division rather than transverse, this fact rather favoring a

protozoal as against a bacterial nature. This spirochaete is from 24–30 microns long, about 0.45 micron broad and has a corkscrew motility. It is readily transmissible to a number of laboratory animals, as monkeys, white rats, etc.

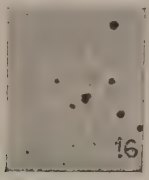


FIG. 111.

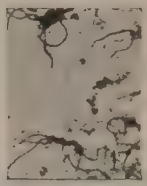


FIG. 112.

FIG. 111.—Showing *Leptospira*. (*After Noguchi in Journal of Experimental Medicine.*)

FIG. 112.—A group of *Leptospira icterohaemorrhagiae* from a culture, stained by Fontana's method. They fail to show their elementary spirals by this staining. (*After Noguchi in Journal of Experimental Medicine.*)

B. berbera.—This, the spirochaete of Northern African relapsing fever, causes the disease as seen in North Africa and Egypt. It is transmitted by lice, Nicolle and others having shown that the spirochaetes make their way from the alimentary tract to the body cavity of the louse. They have shown that the bite alone of an infected louse is innocuous and also that the faeces are noninfective, when injected into monkeys. Emulsions of infected lice, however, when rubbed into wounds, produce the disease in monkeys.

FIG. 113.

The spirochaetes taken in by a louse disappear in a few hours and the insect remains harmless until about the fifth day, when it becomes infectious, and so remains

FIG. 114.

FIG. 113.—Four specimens of *Leptospira icterohaemorrhagiae*. They appear blunt and curved and without any indication of the minute elementary spirals which are the characteristic feature of this genus. (*After Noguchi in Journal of Experimental Medicine.*)

FIG. 114.—A *Leptospira* viewed under the dark-field microscope, showing its minute elementary spirals. (*After Noguchi in Journal of Experimental Medicine.*)

until the twelfth to fifteenth day. Spirochaetes reappear in the coelomic fluid of the louse about the sixth day and continue present until about the twentieth day.

A striking fact is that infection can be brought about a day before spirochaetes appear and that after a period of a few days these spirochaete-containing lice lose

their power to infect. It would seem that the infecting stage was an invisible one. Have we then a symbiosis between a spirochaete and an invisible virus, possibly filterable? Wolbach has shown that certain spirochaetes will pass through a Berkefeld filter as spirochaetes but this would not affect the possibility of the existence of some granule or chlamydozoal stage. It may be that the infecting stage is not an invisible one but a granule one.

It is by crushing the louse, by scratching or otherwise, that the spirochaetes contained in the coelomic fluid reach and penetrate the wound of the bite. This is therefore a contaminative method of infection.

B. carteri.—Mackie has shown that the Indian relapsing fever, which is caused by *B. carteri*, is probably transmitted by the louse, and it is probable that the conditions under which the infection takes place are similar to those occurring with *B. berbera* infections.

B. recurrentis.—With the European relapsing fever, bedbugs have been suggested as the transmitting agents. The probabilities are however that this infection is transmitted by lice alone.

A relapsing fever of Persia is transmitted by a tick of the genus *Ornithodoros*. The relapsing fever of Panama has been shown to be transmitted by the *Ornithodoros talaje*.

Diagnosis.—In blood examinations we may use the dark-field illumination, although the spirochaetes stain readily with Wright's stain. In examining blood for spirochaetes by means of dark-field illumination it is important to know that spirochaete-like filaments (pseudospirochaetes) are apparently produced by protoplasmic extrusions from red cells under the influence of such deleterious agents as heat from platinum loop, etc. The India-ink method is a good one. Hagler recommends smearing out a mixture of one loopful of blood and a collargol preparation made by diluting one part collargol with two parts water, allowing it to stand 24 hours, then filtering.

There is great variation in the description of the different spirochaetes, and frequently measurements are given for short forms and long forms. They also vary from wave-like lines to corkscrew spirals. Again, different species have different types of movement. As a rule they are about 20 × 0.4 microns. Noguchi has cultivated the various species of pathogenic human spirochaetes by employing a method similar to that used in cultivating the organism of syphilis. He noted longitudinal division in his cultures.

Other Blood Spirochaetes.—While it is realized that the organisms of yellow fever, infectious jaundice and seven-day fever, belonging to the genus *Leptospira*, are not so distinctly blood parasites as those of the relapsing fevers, but rather take an intermediate position between them and the true tissue spirochaetes, they will be included in this section.

Leptospira icteroides.—It is now generally accepted that the cause of yellow fever is a spirochaete, *Leptospira icteroides*, which is very similar to but slightly smaller than *L. icterohaemorrhagiae* of infectious jaundice. Noguchi injected 74 guinea pigs each with about 5 cc. of blood from 27 cases of yellow fever. The blood from 6 of these cases proved

infectious, producing in 8 guinea pigs fever, conjunctival injections, albuminuria, a leukocytosis followed by leukopenia and, after a few days, a subsidence of the fever to normal or subnormal. At this time jaundice and haemorrhages occurred.

Autopsies of the guinea pigs showed a fatty and yellow liver with nephritis. Some of the injected guinea pigs showed only fever but seemed to have acquired an immunity to subsequent injections of a virulent virus. In the blood, liver and kidneys of the animals showing the jaundice and albuminuria Noguchi was able to demonstrate spirochaetes by dark-field illumination. He also obtained cultures from such animals. Leptospiras were demonstrated in the blood of 3 out of 27 human cases but only after prolonged search. Cultures have been obtained from human yellow fever blood. For culturing, a medium is used consisting of 1 part of serum and 3 parts of Ringer's solution made semisolid with 0.3% agar and contained in tall

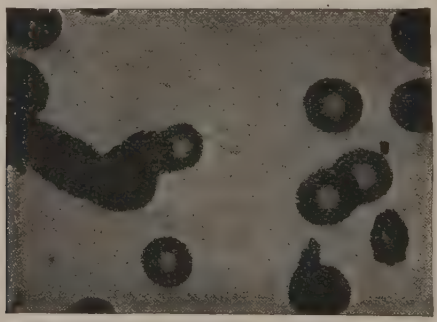


FIG. 115.—*Leptospira icteroides* in blood of guinea pig experimentally inoculated with culture from blood of yellow fever patient. (*After Noguchi in Journal of Experimental Medicine.*)

tubes. One cubic centimeter citrated yellow fever blood is introduced into the lower part of the medium. A thin layer of liquid petrolatum is poured on the top of the medium. We need partial oxygen tension but not anaerobiasis. Optimum growth temperature is 33°C. *L. icteroides* is from 4 to 9 microns long by 0.2 wide and tapers gradually to extremely thin sharp points. These organisms will pass the pores of V and N Berkefeld filters thus placing them in the group of filterable viruses. The virulence of different strains varies, with some strains as little as 0.00001 cc. of culture proving fatal for guinea pigs. Monkeys, rabbits and birds were not susceptible to infection but the marmoset and puppy seemed to respond as did the guinea pig.

By having the mosquito (*Aedes aegypti*) feed on infected guinea pigs as well as the human case of yellow fever, and subsequently allowing these mosquitoes to bite normal guinea pigs, the disease was transmitted in a few cases. In certain infected mosquitoes Noguchi found leptospiral organisms with dark-field illumination. Mosquitoes

fed on infected guinea pigs became infectious in eight days, this shorter period as compared with that following human feedings being due, probably; to the greater abundance of organisms in the blood of infected guinea pigs.

Guiteras doubts the etiological relation of Noguchi's *Leptospira* to yellow fever on the ground that in view of the susceptibility of animals to this organism the disease should exist as an epizootic, whereas its occurrence as an animal epizootic is unknown and is improbable in view of the ease with which yellow fever is eradicated when measures applied solely to man and the mosquito are practised. Among other points of objection he notes the recovery of the spirochaete from the blood later than the third day of the disease.

Leptospira icterohaemorrhagiae.—This spirochaete, the cause of infectious jaundice, is the type species of a genus described by Noguchi

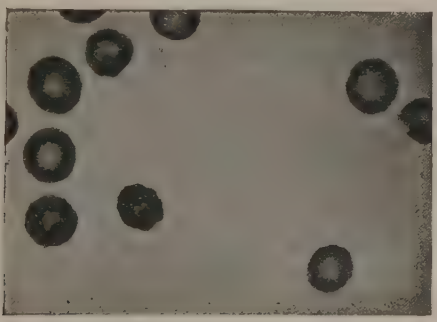


FIG. 116.—*L. icteriodes* in blood of guinea pig from culture from blood of case of yellow fever. (*After Noguchi in Journal of Experimental Medicine.*)

as having minute elementary spirals running throughout the body and failing to show either flagella or undulating membrane.

The constituent spirals are closely placed and the total length may reach 15 to 20 microns. The dark-field illumination is preferable for its demonstration although it shows up well by various staining methods. To culture use a medium of one part of rabbit's serum with three parts of Ringer's solution inoculating with citrated plasma. The organism is found in the blood during the first three or four days of the disease. It is also present in the urine. Young guinea pigs are particularly susceptible and in them we have, following injection of the blood of a case, jaundice, albuminuria and haemorrhages. At autopsy the spirochaetes are best demonstrated in a liver emulsion. Infection with this spirochaete is common among rats in various parts of the world so that it may be considered a natural infection of rats, but they do not seem to suffer from it.

It is considered that the infection is transmitted through the urine of infected rats or men. The spirochaetes may gain entrance through the skin or by mouth. The

fact that the spirochaetes seem to die in urine within 24 hours makes infection by the contaminated urine questionable but experiments with various arthropods have failed to indicate their connection with the transmission of the disease..

Leptospira hebdomadis.—The Japanese have proved one form of seven-day fever to be due to *L. hebdomadis.* The organism has been found in the blood and is excreted with the urine. In Japan it is thought to be carried by a species of field-mouse.

Leptospira morsus-muris.—This organism is the cause of rat bite fever, a relapsing type of fever following the bite of infected rats.

In a study of this disease Futaki and others discovered the spirochaete in the tissues of the bite area and the adjacent lymphatic glands (1915). These spirochaetes were about 10 microns long. In the blood of man and infected animals shorter and thicker spirochaetes are found (3 to 6 microns). When cultivated in the media used for other *Leptospira* we have longer forms up to about 20 microns. The shorter forms are considered as young organisms. The body is rigid and one, two or more flagella may be detected on each end.

Under the dark-field the motion resembles that of vibrios rather than spirochaetes. They stain quite readily. In fact, Noguchi, Wenyon and others are inclined to believe, as Zuelzer previously suggested, that the organism is not a true spirochaete, but rather more of the nature of a *Spirillum.* In this case the name would be *Spirillum morsus-muris,* or as Robertson points out, if the organism in man and rat is identical—*Spirillum minus.*

L. morsus-muris is found in the blood of infected mice, rats or guinea pigs during the first two weeks and then becomes distributed in the connective tissues especially that of the lips, bridge of nose and tongue. They are not secreted in the saliva but the transfer seems to occur by a break in the spirochaete-containing tissues and thus inoculation into the bite wound. The organism may possibly be excreted in the urine.

Tissue Spirochaetes

While it is true that the spirochaetes of syphilis and yaws occur in the blood, as is shown by the infectivity of the blood of patients, their presence there is probably temporary and it is in the tissues, particularly the lymph nodes which apparently constitute an important reservoir of infection, that we find the organisms in such numbers as to be microscopically demonstrable. To such organisms, in which a biological affinity for the tissues is indicated by their customary distribution, I have reserved the name *Treponema;* while for those easily demonstrable in the blood stream the generic name *Borrelia* has been preferred. Previously I have mentioned the morphological distinction between the two genera pointed out by Noguchi—that the spirals of *Borrelia* are flexible and those of *Treponema* rigid.

Treponema pallidum.—In 1905 Schaudinn and Hoffmann first described the occurrence of spirochaetes in the primary lesion and swollen lymphatic glands of syphilis and gave to them the name *Spirochaeta pallida*. This spirochaete is characterized by the geometric regularity of its spirals which are deeply cut and, like a cork-screw, remain sharp when the focus is changed. The organism varies from 3–15μ in length, and from 0.3–0.4μ in width. The spirals are about 1μ apart, so that a spirochaete with eight turns would be about 8μ long.

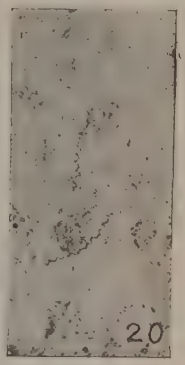


FIG. 117.—*Treponema pallidum*. Stained smears. (*After Noguchi in Journal of Experimental Medicine.*)

The organism is best seen with the dark-field, in which they continue to show a silvery whiteness when we focus up and down while other spirochaetes under such circumstances show a brownish tinge. While its rotary movement is rapid, it does not move across the field with the speed of other spirochaetes. Thus, the *Spirochaeta refringens*, an organism commonly present in genital ulcerations, quickly traverses the field, and—another point of distinction—shows more widely separated spirals. The *pallidum* shows a continuity of its spirals while in motion, but when at rest often presents the appearance of a series of silvery dots or dashes. Many individuals show a bend in the long axis.

The organism is constantly found in scrapings from the primary lesion and is easily demonstrated in many of the secondary lesions. Finding the spirochaetes in tertiary lesions is far more difficult. Noguchi has demonstrated treponemata in all layers of the cerebral cortex except the outermost one in 12 out of 70 cases of general paresis. The organism has also been demonstrated in sections from cases of syphilitic aortitis. In cases of congenital syphilis, the spirochaetes are found in abundance in the liver, and have been demonstrated in the lungs, kidneys and even the heart muscle. Pathologically the most prominent characteristic of the organism is its tendency to invade the perivascular lymph spaces and thence to attack the intima of the smaller blood vessels, obliteration of their lumen resulting. Noguchi has succeeded in inoculating two monkeys with cultures of spirochaetes of human origin, thus demonstrating the causal relation of the organism to the disease.

Animal pathogenicity.—In the chimpanzee a primary sore was followed by secondary lesions after inoculation of material from a human source. Monkeys can be infected by rubbing syphilitic material into cutaneous abrasions of the eyebrows. Rabbits can be inoculated upon the cornea, but they are chiefly used for intratesticular injections of spirochaete-bearing material. The testicle shows an elastic swelling in about two weeks, increasing to about two months. Upon examination, the affected area is found to be teeming with spirochaetes.

Filtration experiments with treponemata seem to show that the filtrate is non-infectious; hence the organism appears not to show in any stage a form in which it is filterable.

Cultivation.—*T. pallidum* has been cultivated anaerobically in horse serum by Schereschewsky. The cultures contained other organisms. Muhlens, by growing anaerobically on horse serum agar (1 to 3), claims to have obtained pure cultures. Animal inoculations with this material were negative, however.

Noguchi has cultivated *T. pallidum* under strict anaerobic conditions in a medium of ascitic fluid containing a piece of fresh sterile tissue, preferably placenta. The growth is faintly hazy and does not have an offensive odor. *Spirochaeta microdentium* shows similar morphology but the cultures have a foul odor. *Sp. macrodentium* is similar culturally but differs morphologically.

Luetin.—When cultures of *T. pallidum*, grown for one or more weeks in ascitic fluid agar and ascitic fluid, are ground in a mortar, heated to 60°C. for one hour, then, with the final addition of 1% trikresol, we have an emulsion called "luetin." This extract produces an allergic reaction on the skin of certain syphilitics (luetin reaction). To carry out the test luetin is introduced intradermally at the insertion of the left deltoid and a control emulsion of agar media injected in the right arm. A negative result shows as an erythema without pain or papule formation. Positive

FIG. 118.

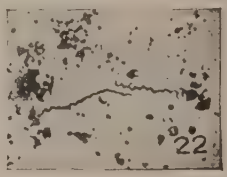


FIG. 119.

FIG. 118.—*Treponema pallidum.* Dark-field. (*After Noguchi in Journal of Experimental Medicine.*)

FIG. 119.—*Treponema pallidum.* Fontana's stain. (*After Noguchi in Journal of Experimental Medicine.*)

reactions show as papules, vesicles or even pustules giving rise to discomfort for several days. Not only do we have papular and pustular type reactions but also torpid ones (taking ten days or more to develop). While the control side usually becomes normal in 48 hours yet in latent and tertiary syphilis the control may show almost as marked a reaction as occurs in a positive one. The term "Umstimmung" is applied to this susceptibility to trauma of the skin of those having tertiary syphilis. Some cases of parasyphilitic infections which are negative to the Wassermann test give a positive luetin reaction. Tertiary yaws cases frequently give a positive luetin reaction. See comparison of Wassermann and luetin statistics.

Test of curability.—Pearce and Brown demonstrated that *T. pallidum* localized in lymph nodes of the experimental rabbit, and remained viable and virulent for four years or more. This has been repeatedly confirmed. Engman and Eberson demonstrated the infectiousness of lymph nodes of patients with latent syphilis in whom infection was of several years standing (maximum probably eleven years). Nichols and Walker applied the study of infectiousness of lymph nodes of syphilitic rabbits after treatment to the evaluation of antisyphilitic agents, and showed that these structures were rendered non-infectious by the use of arsphenamine. The applica-

tion of tissue-transfer method to the study of the problem of curability of syphilis in the human suggests itself (Chesney and Kemp). Lymph node sterility after treatment does not necessarily indicate complete absence of infection, yet this is probably a more valid test of curability of syphilis than is the reinoculation test (reinfection of a treated animal being taken as proof of cure).

Diagnosis.—In examining material for diagnosis, use either the dark-ground illuminator or a stained smear. To obtain serum for examination for treponemata, wash the lesion with alcohol; dry, and rub with gauze, or gently scrape with a scalpel, so that lymph from the corium may be obtained. Blood should be gently wiped away and the specimen made from the lymph that eventually exudes clear. The serum may also be obtained by washing the lesion, and, while the surrounding surface is still wet, applying a Bier cup or a test tube in which a partial vacuum has been created by flaming. The smears should be thin, and spread on scrupulously clean slides.

Fontana's silver staining method is excellent. The India-ink method of Burri also is highly recommended. Take one loopful of secretion from a chancre and deposit it on one end of a slide. Emulsify in this a small quantity of drawing ink as much as can be obtained by touching the end of a new spatulate toothpick in the ink. Use the toothpick for mixing. I use the method of sticking a fine glass pipette into the underlying corium of the chancre and get serum in that way or by squeezing the chancre afterward. Mix and make a smear as for blood. When dry examine with the oil-immersion objective and the treponemata will be found to stand out as white spirals against a dark background. Treponemata often appear as if bent in the middle.

Harrison prefers collargol to India ink. One part of collargol is put in a bottle with 19 parts of water and well shaken. This shaking is repeated. One loopful of the suspected serum and one loopful of the collargol suspension are mixed and smeared out and examined as for the India-ink method. Benian's method using Congo red stain gives excellent results. See page 67.

For Warthin and Starry's method see page 68.

The subject of the complement fixation and precipitation tests of the blood serum is discussed under "Immunity."

In the diagnosis of cerebrospinal syphilis we use, in addition to the complement fixation and precipitation tests of the blood: (1) The globulin increase reaction in which about 1 cc. of a saturated aqueous solution of ammon. sulphate is added to an equal amount of cerebrospinal fluid. If turbidity or rather opalescence appears immediately, or within three minutes, the test is positive. We now use a ring test. (2) The counting of the lymphocytes in the cerebrospinal fluid. A lymphocytosis occurs in cerebrospinal syphilis, tabes and general paresis. (3) The Wassermann or Kahn test, using the cerebrospinal fluid instead of blood serum. (4) The colloidal gold test of Lange. These various examinations of spinal fluid are taken up in detail in chapter on cytodiagnosis and spinal fluid examinations.

T. pertenue.—This organism was first reported as present in yaws by Castellani. It resembles *T. pallidum* in morphology and general distribution.

A point of distinction between these spirochaetes is that the *T. pallidum* is found in abundance in sections from a chancre about the thickened arteries in the corium, while in sections from a yaws nodule the *T. pertenue* is found chiefly in the region of the interpapillary pegs of the Malpighian layer of the epidermis where they bound the papillary layer of the corium.

The organism is constantly found in the unbroken early lesions of yaws and may be found in lymphatic glands. Injection of blood of yaws cases into monkeys brings about infection but the spirochaete has not been demonstrated microscopically in the blood. The spirochaete is not apt to be found in the tertiary lesions.

T. pertenue has been cultivated in the same way as *T. pallidum* and Nichols has infected rabbits by intratesticular injection. A disease of Guam known as gangosa is generally regarded as a tertiary form of yaws. In persons who have had yaws a positive Wassermann reaction seems to be given in a higher percentage than is true for syphilis. Arsphenamine is also more specific for yaws than for syphilis.

Other Spirochaetal Organisms.—An organism about which a great deal has been written in connection with confusing it with the organism of syphilis is *Spirochaeta refringens*. It is longer and thicker than the spirochaete of syphilis and has several irregular, wide spirals. It moves more quickly than *T. pallidum*. It stains quite readily. It has been found in the mouth but it is best known in connection with genital chancres. There are numerous spirochaetes found normally in the mouth such as *Spirochaeta buccalis*. Some of the buccal spirochaetes belong to the treponemata. A well known mouth spirochaete is that found in symbiosis with Vincent's fusiform bacillus. It has been thought by some to be a stage in the development of the fusiform bacillus. Spirochaetal organisms have also been found associated with amoebae in dental affections. Spirochaetes have been described in connection with a not uncommon affection, resembling pulmonary tuberculosis known as bronchial spirochaetosis.

Free living spirochaetes have been cultivated from tap water in our laboratory and by various other workers.

CHAPTER XVIII

FLAT WORMS

Classification of the Platyhelminthes (Flat Worms)

Class Trematoda

Superfamily	Family	Genus	Species
Paramphistomoidea	Gastrodiscidae	Gastrodiscus	G. hominis
	Paramphistomidae	Watsonius	W. watsoni
Fascioloidea	Fasciolidae	Fasciola	F. hepatica
		Fasciolopsis	F. buski
	Troglotrematidae	Paragonimus	P. ringeri
	Echinostomatidae	Echinostoma	E. ilocanum
	Opisthorchiidae	Opisthorchis	O. felineus
		Clonorchis	C. sinensis
	Heterophyidae	Heterophyes	H. heterophyes
		Metagonimus	M. yokogawai
	Dicrocoeliidae	Dicrocoelium	D. lanceatum
Schistosomatoidea	Schistosomatidae	Schistosoma	S. haematobium
			S. japonicum
			S. mansoni

Class Cestoda

Bothriocephaloidea	Diphyllobothriidae	Diphyllobothrium	D. latum
		Diplogonoporus	D. grandis
Taenioidea	Davaineidae	Davainea	D. madagascariensis
	Dipylidiidae	Dipylidium	D. caninum
	Hymenolepididae	Hymenolepis	H. diminuta
			H. nana
	Taeniidae	Echinococcus	E. granulosus
		Taenia	T. solium
			T. saginata

Note.—Two larval Taeniidae are found in man (*Cysticercus cellulosae* and *Echinococcus granulosus*); also two larval Diphyllobothriidae (*Sparganum mansoni* and *Sparganum proliferum*).

Two parasites often referred to as ophthalmic flukes have been reported lying between the crystalline lens and its membrane. They have been considered as possibly trematode larvae. *Agamodistomum ophthalmobium* was found in 1850 in the eye of a child and *Monostomulum lentis* in the eye of an old woman.

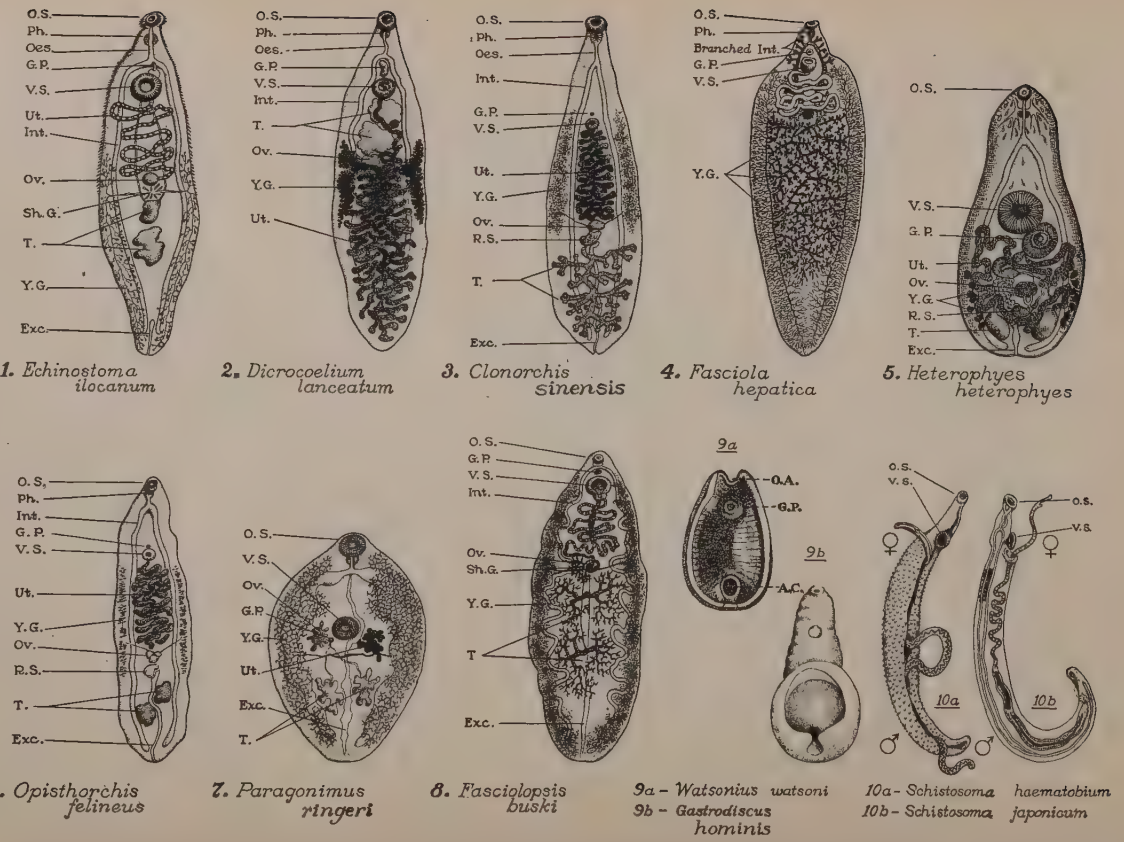


FIG. 120.—Anatomy of trematoda (flukes) of man. *O.S.*, oesophageal sucker; *Ph*, pharynx; *Oes*, oesophagus; *G.P.*, genital pore; *V.S.*, ventral sucker or acetabulum; *Ut.*, uterus; *Int.*, intestines; *Ov*, ovary; *Sh. G.*, shell gland; *T.*, testicles; *T.G.*, yolk glands or vitellaria; *Exc.*, excretory pore.

Trematodes or Flukes

Flukes are generally leaf-like in outline, rarely cylindrical, and exhibit marked variation in size and shape. They are nonsegmented and possess a mouth and a pharynx. Very characteristic of them is the possession of suckers by which they hold on to the skin or mucosa of their host.

They are divided into two subclasses, (1) the Monogenea in which the egg gives rise to a larva which later becomes the adult without an intermediate host and (2) the Digenea. It is to this latter that the flukes parasitic in man belong. This subclass is characterized by the fact that the larva becomes parasitic in some intermediate host and there gives rise to a second generation of larvae which latter develop into adults in the vertebrate host.

The largest human fluke, *Fasciolopsis buski*, is from 2 to 3 inches (50 to 75 mm.) in length, while the *Heterophyes heterophyes* is less than $\frac{1}{12}$ inch (2 mm.) in length. The most important fluke, the liver fluke, *Clonorchis sinensis*, is flat and almost transparent, while the almost equally important lung fluke, the *Paragonimus ringeri* is oval, almost round and reddish-brown in color. With the exception of the Schistosomatidae, all flukes are hermaphroditic, and, with the exception of this family, all flukes have operculated eggs. The only other operculated (with a lid) eggs we meet with in man are those of the Diphyllobothriidae.

Classification.—The three important superfamilies of flukes parasitic for man are:

1. *Paramphistomoidea*—flukes with two suckers situated at either extremity.

2. *Fascioloidea*—flukes with two suckers, one terminal, the other adjacent to it and situated ventrally. This family includes the important genera *Fasciola*, *Opisthorchis*, *Dicrocoelium*, *Fasciolopsis*, *Paragonimus*, *Clonorchis*, *Heterophyes*, *Metagonimus* and *Echinostoma*.

In *Paragonimus* and *Heterophyes* the genital pore is posterior to the acetabulum, in the other genera it is anterior. *Fasciola* has a dendritic intestinal canal which is not the case with *Clonorchis*, *Fasciolopsis*, *Echinostoma* (*Fascioletta*), *Opisthorchis* and *Dicrocoelium*. In *Dicrocoelium* the testicles are anterior to the uterus, in *Opisthorchis*, *Clonorchis*, *Fasciolopsis* and *Echinostoma* they are posterior. *Fasciolopsis* and *Clonorchis* have branched testicles (the former a very large fluke—*Clonorchis* of medium size) while those of *Opisthorchis* are lobed.

3. *Schistosomatoidea*—in this superfamily we have a leaf-like male which by an infolding of its sides makes a channel for the thread-like female. The sexes are separate, not hermaphroditic as with the Fascioloidea and Paramphistomoidea.

Anatomical Considerations.—Flukes have two suckers which, except in the Paramphistomoidea, are quite near each other—one is termed the oral sucker and the other the ventral sucker or acetabulum. The intestinal tract consists of a pharynx, proceeding from the oral sucker, which bifurcates and terminates in blind intestinal caeca. In Schistosomatidea the gut branches reunite and end in a single caecum.

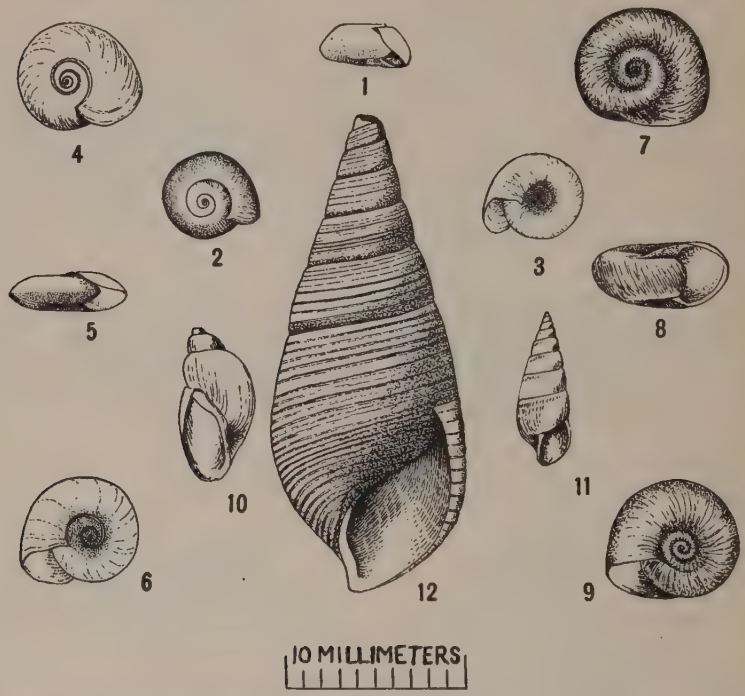


FIG. 121.—Mollusk hosts of human trematodes. 1–3, *Segmentina;* 4–6, *Hippeutis;* 7–9, *Planorbis boissyi* Potiez and Michaud; 10, *Isidora contorta;* 11, *Katayama nosophora* A. Adams; 12, *Melania libertina* Gould. (*By Courtesy of Paul Bartsch.*)

The excretory system takes its origin from the so called flame cells (readily seen in living specimens, especially larvae) in the form of small canaliculi which empty into the two collecting ducts. These unite at the posterior extremity forming the excretory vesicle.

The genital system is of importance for the purpose of differentiation of the various genera. All flukes except the Schistosomatidae are hermaphroditic. The male organs consist of two testes of various shapes. The vasa deferentia unite to form the seminal vesicle which opens at the genital pore, in most genera located at the bifurcation of the gut. The female organs consist of the ovary, oviduct, receptaculum

seminis, yolk glands, yolk collecting ducts, shell gland and uterus. The ovum passes from the ovary through the oviduct, is then fertilized by the spermatozoa from the receptaculum seminis and supplied with yolk cells through the yolk collecting ducts coming from the yolk glands. The shell gland located at the beginning of the uterus proper supplies the resistant egg wall. The ova are now passed along the winding uterus and are finally expelled through the common genital pore.

A canal known as Laurer's canal, leads from the oviduct to the exterior. Its precise function is not known. According to some it takes care of the overflow from the oviduct.

Life History.—The life history of many of the human flukes is now known. The history of others, which is not now known, probably resembles that of the common liver fluke of sheep (sheep rot). The eggs as passed in the faeces of the host are composite, i.e. the ovum proper is surrounded by yolk cells necessary for its development. The eggs reach water and during the course of 2–3 weeks if the available oxygen is plentiful and the temperature favorable the ovum segments forming the mulberry mass. In 3–6 weeks the embryo, the miracidium, is fully formed. It is a ciliated structure without alimentary canal having only a primitive body space. In its anterior extremity the miracidium of the liver fluke is supplied with a solid organ, the proboscis. When the nutriment is exhausted the embryo pushes up the operculum and is set free in the water where it swims about actively. It can live for 24 hours in the free living state but is then in need of a host. When the miracidium reaches some suitable mollusk (*Lymnaea truncatula*) it attacks the tentacles of the snail and by means of the proboscis works through the soft tissue of its new host. It finally reaches the pulmonary chamber of the snail, loses its cilia and becomes a bag-like structure, the sporocyst. From the internal wall of this structure small bodies (masses of cells) form. These masses undergo differentiation, enlarge in size and show the formation of a simple alimentary canal and then are known as rediae. The rediae tend to break out of the sporocyst and wander to the liver of the snail. These rediae may give rise to a second generation of rediae.

From the rediae minute little worms resembling adult flukes in possessing suckers, and a bifurcated alimentary canal, but differing in the possession of a tail, develop (cercaria). Having reached maturity, these cercariae leave the mollusk, and, as in case of *Fasciola hepatica*, lose the tail, become encysted on blades of grass, to be eaten by sheep and again commence the cycle. The encysted cercariae develop into adult liver flukes. It is probable that with many flukes the cercariae enter some host, as mollusk, insect, or fish, and that it is by eating such animals as food that man becomes infected. It has been shown that certain mollusks form the intermediate hosts in schistosome infections, cercariae from these mollusks entering the skin of man. With the lung fluke there would seem to be a primary mollusk intermediate host and a secondary one in certain crabs, which latter, when eaten raw bring about the infection. The idea in China is that the infection with the common liver fluke of man is brought about by eating fish. Fluke disease is generally known as *distomatosis* or *distomiasis*.

Mollusk hosts.—In those trematode infections of man, of which we know the life history, some genus of mollusk serves as an intermediate host. The phylum Mollusca includes unsegmented animals, usually contained in a calcareous shell and made up of visceral mass, head, mantle and foot. The mollusk hosts of trema-

todes belong to the class, Gastropoda, which are known as snails and usually have a spirally coiled shell, a distinct head and a broad flat foot. The mollusks concerned in transmitting fluke diseases have a foot flattened ventrally and are creeping forms (Platypoda). Gastropods with a fin-shaped foot are free swimming forms (Heteropoda). The following are the more important flukes of man with some of the genera of mollusk hosts which have been demonstrated or suspected. *F. hepatica* (*Lymnaea* and *Physa*); *F. buski* (*Segmentina* and *Hippeutis*); *P. ringeri* (*Melania*); *O. felineus* (*Dreissena*); *C. sinensis* (*Bythinia* and *Melania*); *M. yokogawai* (*Katayama* and *Melania*); *D. lanceatum* (*Planorbis*); *S. haematobium* (*Bulinus* (*Isidora?*), *Lymnaea*, *Physopsis* and *Planorbis*); *S. japonicum* (*Katayama* and *Oncomelania*); *S. mansoni* (*Planorbis*, *Physopsis* and *Ampullaria*).

Lymnaea, a host of *F. hepatica*, is a common snail of ponds. It has a delicate fragile elongated shell with a pointed spire.

Melania, which is the primary intermediate host of *P. ringeri*, has a turreted shell with an acute apex.

Katayama, an intermediate host of *S. japonicum*, has a cornucopia-like shell with a blunt apex.

Planorbis, an intermediate host of *S. mansoni*, has a rather thick shell rolled in a flat spiral. The spire of the shell is in one plane.

Bulinus, a host of *S. haematobium*, has an elongated spire with a pointed apex.

Experiments with infection of snails by miracidia show that these avoid unsuitable hosts and attack only the known efficient hosts. It is now known that various species of a genus will act as hosts. In connection with the possible establishment of schistosome infection in the snails of the United States, through eggs from disease-carrying orientals, Cort has been impressed with the differences in the mollusk hosts of *S. japonicum* (*Katayama*) and of the other schistosome infections. Mollusk hosts are not as limited as we formerly regarded them. According to Bartsch *Katayama* and *Blanfordia* differ in habits in that the former requires water of an acid reaction for growth while the latter requires water of an alkaline reaction. *Segmentina* and *Hippeutis* belong to the subfamily Planorbinae. The relationship of *Bulinus* and *Isidora* is under consideration.

Liver Flukes

Fasciola hepatica (Distomum hepaticum).—This fluke, while of enormous economic importance by reason of destruction of sheep, has been reported only 50 times in man up to 1913, and in these instances does not seem to have occasioned marked symptoms.

It has a cone-shaped anterior projection and is about 1¼ inches (30 mm.) long. The intestinal canal, as well as the testicles, is branched. These intestinal diverticula are well marked in the cone just after the branching from the oesophagus. Diameter of acetabulum about 1.6 mm., of oral sucker 1 mm. There is a possible importance of *F. hepatica* in connection with a peculiar affection known as "halzoun." This results from the eating of raw goat-liver, and it is supposed that the flukes crawl up from the stomach and, entering the larynx or attaching themselves about the glottis, produce the asphyxia characteristic of the disease. It is more probable that

they attach themselves to the pharyngeal mucosa in the act of swallowing and do not enter the stomach where the gastric juice would destroy them. The life history of this fluke has been described on page 453. The intermediate host is a species of *Lymnaea*.

Dicrocoelium lanceatum (D. dendriticum).—This has been reported only several times in man. The symptoms are unimportant. The fluke is about ⅓ inch (8 mm.) long, with testicles anterior to the uterus.

Clonorchis sinensis (Clonorchis endemicus).—This fluke is the most important of the human liver flukes and until recently was known as *Opisthorchis sinensis*.

Looss separated this genus from *Opisthorchis* principally by the characteristic of branching testicles—those of *Opisthorchis* being lobed. This fluke is very common in China and Japan—in certain sections of Japan 20% of the population being infected. This fluke is about 10 to 20 mm. long. When squeezed out of the thickened bile ducts it is so transparent and glairy as almost to resemble glairy mucus. As many as 4000 of these parasites have been found in a case, chiefly in the liver, but at times in the pancreas. This fluke is supposed to produce most serious symptoms, as indigestion, swelling and tenderness of liver, ascites, oedema, and a fatal cachexia. As a matter of fact, many physicians in China attribute very little pathogenic importance to it. The disease is diagnosed by the presence of the ova in the stools. The source of infection is probably through the eating of uncooked fish.

FIG. 122.—*Clonorchis sinensis*. (*Jeffreys and Maxwell.*)

Kobayashi has examined various mollusks and fish for trematode larvae. He succeeded in infecting nine kittens and two cats by feeding them with certain freshwater fishes whose flesh contained trematode larvae. These fish were found in districts where human distomiasis was common. Later he fed thirty cats on various cyprinoid fishes whose muscles contained encysted larvae. In a series of dissections he has found that the ingested larval flukes have reached the bile ducts within fifteen hours and in twenty-six days they have attained maturity and begun giving off ova. Species of *Bythinia* and probably *Melania* act as primary intermediate hosts and the fish is the secondary intermediate host.

Opisthorchis felineus.—This fluke is smaller than the *C. sinensis*, and is a common parasite of the gall bladder and bile ducts of cats. There are two-lobed testicles in this species instead of dendritic ones as in *C. sinensis*. In certain parts of Siberia the parasite is found in more than 6% of the human autopsies. The symptoms are similar to those caused by *C. sinensis*. The primary intermediate host is a mollusk, possibly *Dreissena polymorpha*. Certain fish are secondary intermediate hosts. These are eaten raw by natives.

Other liver flukes of less importance which have been reported for man are:

1. *Opisthorchis noverca*. This was found in bile ducts of two natives of Calcutta. It was lancet-shaped and covered with spines. It measures ⅓ by 1/10 of an inch (10 × 2.5 mm.). The eggs are 34 by 21μ.

2. *Metorchis truncatus.* This is a small fluke, 1/12 inch (2 mm.) long, squarely cut across at its posterior end and covered with spines. This was possibly found once in man.

Intestinal Flukes

Watsonius watsoni (Amphistomum watsoni).—This fluke is about 1/3 inch (8 mm.) long, of oval outline but broader at posterior end and has an indistinct oral sucker and a large sucker at the other end. This parasite has been reported from northern Nigeria and is said to be a common infection of regions about Lake Chad. Eggs, 130 × 75μ. In the single reported case the parasites were passed in the stools. The symptoms were a watery diarrhoea.

Gastrodiscus hominis (Amphistomum hominis).—This fluke is about 1/4 inch (6 mm.) long and has a disc-like concavity, about 1/6 inch in diameter from which proceeds a teat-like projection, bearing an oral sucker. The acetabulum is in the posterior border of the disc. While it has been reported only a few times for man, indications are that it is probably fairly common in India and Assam. Eggs, 150 × 72μ. It gives rise to dysenteric symptoms.

Fasciolopsis buski (Distomum crassum).—This is probably a rather common parasite in India, as Dobson found the eggs in 1% of the stools of more than 1000 coolies. Goddard states that more than 5% of stools examined in Shaohing, China, show eggs of this parasite. It is also rather common in Cochin-China. It causes gastrointestinal disturbances. The fluke is from 2 to 3 inches (40 to 70 mm.) in length and about 1/2 inch (12 mm.) in breadth. It is thick, brown in color, and has a very large acetabulum, three to four times the size of the oral sucker and located almost adjacent to it. The branched ovary and shell gland lie in the center with the branched testicles posterior. This species is characterized by a very long and prominent cirrus. The coiled uterus is anterior to the testicles. The eggs measure from 80 to 120 microns, are nearly colorless and have a thin shell with a very small operculum. Nakagawa notes that the eggs hatch in two to three weeks. Certain mollusks of the subfamily Planorbinae particularly species of *Segmentina* and *Hippeutis* serve as intermediate hosts. Infection of man occurs by ingestion of water plants to which encysted cercariae are attached. *F. rathouisi* is now considered to have been a shrunken *F. buski*, as it seems to be anatomically similar to *F. buski*. Kwan's fluke reported from Hong Kong was possibly *F. buski*.

Heterophyes heterophyes (Cotylogonimus herterophyes).—This exceedingly small fluke (2 × 0.5 mm.), which can be recognized by its small size (less than 1/12 inch long) and large, prominent acetabulum, was formerly supposed to be rare. The oral sucker is much smaller than the acetabulum. The elliptical testicles lie at the extreme posterior end. Cuticle has scale-like spines. The eggs are 30 × 17μ. Very characteristic of this genus is the large sucker-like genital pore just below and to one side of the acetabulum and surrounded by a collar of spines. Looss has shown that it is quite common in Egypt, he having found it twice in Alexandria in nine autopsies. The parasites occupy the ileum. It is common in dogs. Mollusk host unknown. Man acquires the infection by eating raw fish—*Mugil cephalus*.

Heterophyes nocens.—This is a small fluke found in the middle part of the small intestine of man. It is common in Japan where the infection is due to the habit of eating raw fish. Examination of fish, especially the species *Mugil cephalus*,

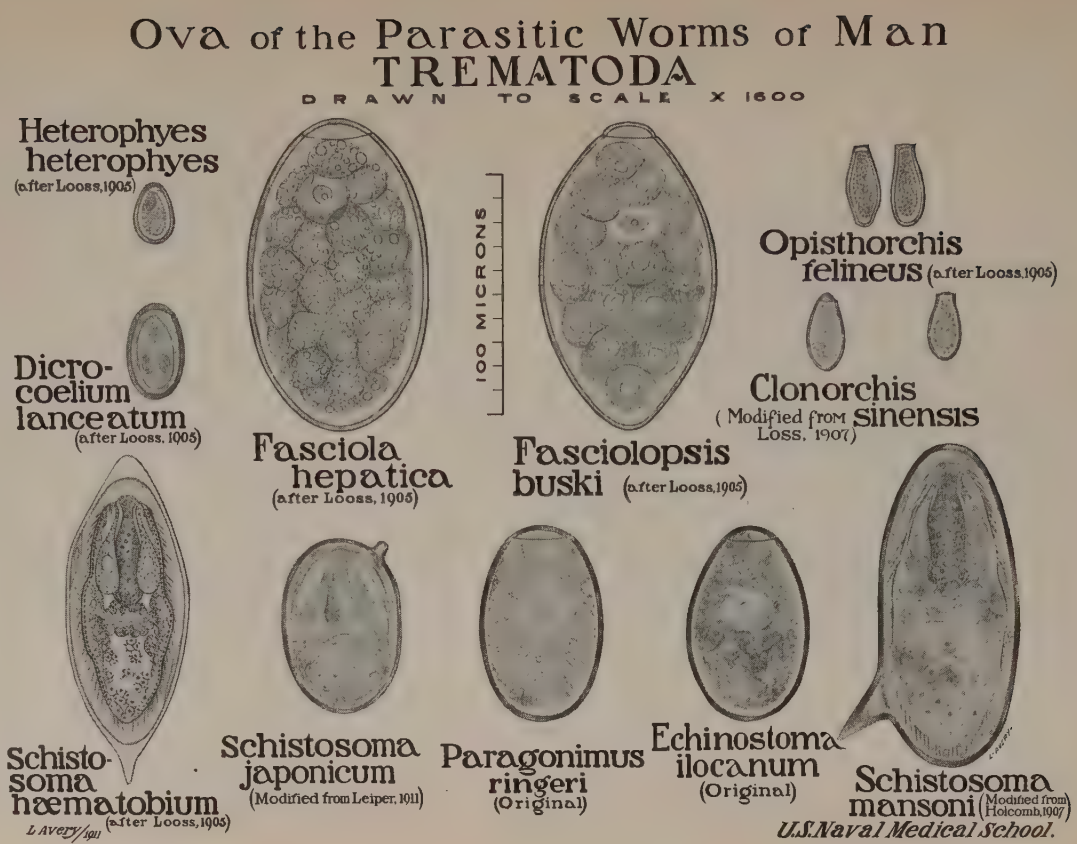


FIG. 123.—Trematode ova.

showed the presence of many encysted cercariae. Experimental animals were easily infected by feeding them with fish containing the encysted larvae. Some consider *H. nocens* the same species as *H. heterophyes*.

Echinostoma ilocanum (Fascioletta ilocana).—This is a small fluke, about ¼ inch (6 mm.) long. There are two massive testicles in the posterior part of body. The acetabulum is prominent and about 500μ in diameter. This fluke has a ring of spines around the anterior extremity. Ovary anterior to testes. Genital pore anterior to acetabulum. The egg of this small fluke is quite large (100μ) and has an operculum. These trematodes were found by Garrison in five natives of Luzon, P. I., after treatment with male fern. Hilaris subsequently reported five new cases. He believes that it is a common fluke of Zambales. Another intestinal fluke reported from Tamils in Malay States is *E. malayanum*. It is about ½ by ⅛ inch (12 × 3 mm.).

Metagonimus yokogawai (Loxotrema ovatum).—This is a fluke of the small intestines and is about the same size as *Heterophyes*. The acetabulum is displaced to the side together with the common genital pore. The latter is not surrounded by a collar of spines. It is a parasite of Korea and Japan. The cercariae which develop in the liver of *Melania* enter fish *Plecoglossus altivelis* which when eaten raw cause the infection.

The eggs of this fluke are elliptical and measure 30 microns in length. Muto states that the cercariae bore into the flesh of gold fish and encyst. He was unable to infect animals with such cysts prior to the end of the third week after encystment. This is proof of the need of a second intermediary host.

Lung Flukes

Paragonimus ringeri (Distoma ringeri, P. westermani).—In certain parts of Japan and Formosa it is estimated that as many as 10% of the inhabitants may harbor this parasite.

It is also common in China, and recently many cases have been reported in the Philippines.

It is popularly known as endemic haemoptysis on account of the accompanying symptoms of chronic cough and expectoration of a rusty-brown sputum. After violent exertion, and at times without manifest reason, attacks of haemoptysis of varying degrees of severity come on. The characteristic ova are constant in the sputum and establish the diagnosis. The fluke itself is a little more than ⅓ inch (8 mm.) long and is almost round on transverse section, there being, however, some flattening of the ventral surface. The acetabulum is conspicuous and opens just anterior to the middle of the ventral surface. Eggs about 90 × 65μ.

The testicles and ovary are branched. The testicles are situated behind the laterally placed ovary. The uterus is placed on the opposite side of the ovary and the genital pore opens just below the acetabulum.

It is rather flesh-like in appearance and is covered with scale-like spines. The flukes are usually found in tunnels in the lungs, the walls of which are of thickened connective tissue. There may be also cysts formed from the breaking down of adjacent tunnel walls. In addition to lung infection with this fluke, brain, liver, and

intestinal infections may be found. Musgrave was the first to call attention to the frequency of general infection with this parasite (paragonimiasis) in the Philippines. He found it in 17 cases in one year.

Nakagawa has found that the miracidia infest certain fresh-water mollusks (species of *Melania*) and become cercariae in this first intermediate host. From

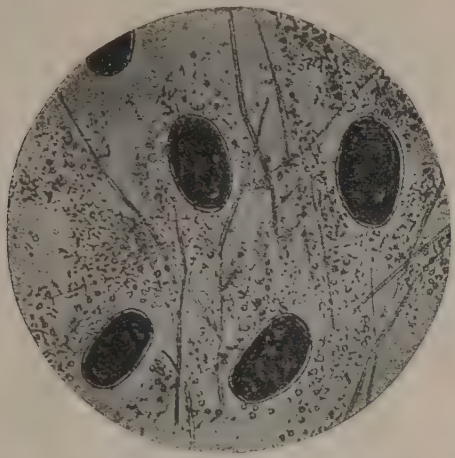


FIG. 124.—Sputum of man containing eggs of the lung fluke, greatly enlarged. (*After Manson.*)

this host the cercariae go to certain fresh-water crabs and encyst in this second intermediate host, either in the liver or in the gills. In Japan one of these crab hosts, *Potamon dehaani*, is eaten both raw and cooked.

Experimental feeding of puppies on infected crabs brought about infection with the lung fluke. It is thought that the fluke, after leaving the cyst, goes through the intestine to the abdominal cavity. Thence it perforates the diaphragm and enters the pleural cavity, finally penetrating the lung to become encysted there. The lung is the favorite site but wandering flukes may invade other tissues and organs even invading the central nervous system.

FIG. 125.—*Paragonimus ringeri* natural size; at left showing ventral surface; at right showing dorsal surface. (Braun after Katsurada.) (*From Tyson.*)

It takes 3 or 4 weeks for the development of the miracidium in the egg. In colder weather it may take twice as long. Crayfish serve as the second intermediary host in Korea.

Besides man, dogs, cats and especially hogs may be infected. The species occurring in hogs in the U. S. is *P. kellicotti*. *P. westermani* is a parasite of the tiger. The question of the identity of *P. ringeri* and *P. westermani*, according to Stiles, should be restudied.

Kobayashi states that in Korea there are no specific differences in the lung flukes of man, dog or hog.

Another fluke which has been reported from the lung is *Fasciola gigantica* (very similar to *F. hepatica*). This was coughed up by a French officer who has been in Africa.

Blood Flukes

The most important of the human flukes are those found in the blood vessels. Such infections are exceedingly common in Egypt and certain areas of the Orient and are of importance in the West Indies and South Africa. The disease is often called bilharziasis, after Bil-

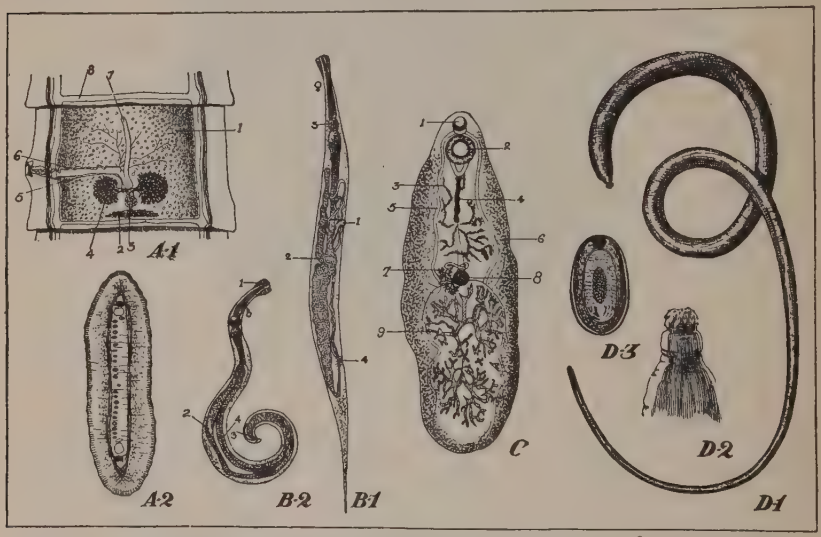


FIG. 126.—Illustration showing anatomical distinctions between a cestode, a nematode, a trematode and one of the Acanthocephala. A1. *Taenia saginata;* 1. Testicles; 2, Yolk glands; 3. Shell glands; 4. Ovaries; 5. Vagina; 6. Vas deferens; 7. Uterus; A2. Cross section of same. B1. Female *Enterobius;* 1. Vulva; 2. Ovary; 3. Bulb oesophagus; 4. Anus; B2. Male *Enterobius;* 1. Oesophageal inflation. C. *Fasciolopsis buski.* 1. Oral sucker; 2. Acetabulum; 3. Uterus; 4. Cirrus pouch; 5. Intestines; 6. Yolk glands; 7. Ovary, 8. Shell gland; 9. Testicles. D. 1, 2, 3. Worm, head and egg of *Macracanthorhynchus hirudinaceus.*

harz, who in 1851 first associated the parasite and the disease. A better name is schistosomiasis, as the three parasites which give rise to different clinical conditions belong to a common genus *Schistosoma.*

The schistosomes differ from other flukes in many respects. They are sexually separate instead of hermaphroditic. The gut branches unite to form one caecum. Pharynx is absent. The eggs do not have an operculum.

Life History.—A ciliated embryo, the miracidium, breaks out of the egg shell when the urine or faeces containing such eggs is diluted with water and in certain

snails forms a sporocyst from which are developed forked-tailed cercariae without a pharynx. These escape from the snail and swimming about in the water penetrate the skin of any one wading or bathing in such infested water. They may also penetrate mucous surfaces so that one may become infected by the drinking water, the cercariae penetrating the buccal mucosa. The cercariae are killed by gastric juice. There is an idea that the preputial mucous membrane is a favorite site of entrance. After penetrating the skin or mucous membrane the cercariae enter the veins to be carried to the right heart and lungs whence they make their way to the liver and tributaries of the portal vein, developing there into males and females. The males and females remain separate until maturity when the shorter, flattened male becomes joined to the longer filiform female by an infolding of the sides, so that the female is enclosed in a canal called the gynaecophoric canal. Thus united, the male carries the female against the portal current to the mesenteric veins whence eggs are given off to appear in the bladder or intestinal tract. Catto considers that *S. japonicum* may live in both arteries and veins. The other two species live only in branches of the portal vein. The male of these blood flukes is about ½ inch (13 mm.) long. The female is longer than the male (about ⅝ inch long) and is thread-like and of a darker color, her two extremities projecting from the canal of the male in which she lives. The ovary lies anterior to the union of the gut branches. The oral sucker of the male is infundibuliform and is smaller than the pedunculated acetabulum. In the female the oral sucker is larger than the acetabulum. The above general outline of life history holds for all these human schistosomes.

FIG. 127.—*Schistosoma japonicum* (male and female). The sharp edges of the borders at the beginning of the gynaecophoric canal formed by the male are an accidental appearance. (*From Mense.*)

Schistosome Species.—While various schistosomes are known for other animals we have for man three species of the genus *Schistosoma: S. haematobium, S. mansoni* and *S. japonicum.* The first two have eggs with sharp spinous processes which are lacking for the eggs of the latter or Oriental species. Then too the dorsal surface of the male of the Oriental species is smooth while that of the other two species is dotted with small rough tubercles.

Schistosoma haematobium.—The male of this species has a tuberculated cuticle and 4 or 5 large testes. The thread-like female has the ovary in the posterior part of the body. The male is about ½ inch long and although really flat appears round from the folding in of the sides. The oral and ventral suckers are close together. The female is almost an inch in length with a long uterus, ovary in the posterior half of the body and the vitellaria in the posterior fourth. In both sexes the gutbranches reunite in about the center of the body. The eggs of this species show terminal spines. The egg-bearing female gives off these eggs in the veins of the bladder although rarely such eggs may be found in the rectal mucosa. As a result of the penetration of the vesical veins and walls of the bladder we have the development of haematuria. In the bloody urine we find the terminal-spined eggs. Inflammation of the bladder ensues and the eggs may form a nucleus for calculus formation. The disease is called vesical schistosomiasis or endemic haematuria. Transmission of the disease is mainly through snails of the genus *Bulinus* (*Isidora?*) in Egypt and South Africa and *Planorbis* in Portugal.

Schistosoma mansoni.—The male of this species has a tuberculated cuticle. So closely do the male and female resemble *S. haematobium* that for a long time Looss held that there was no distinction of species, notwithstanding the fact that it had lateral-spined eggs and gave rise to a rectal type of bilharziasis. Leiper has shown that the male has 8 small testes and that in the female the uterus is short and the ovary in the anterior half of the body. In both sexes the gutbranches reunite in the anterior half of the body. Miracidia from these eggs avoid snails of the genus *Bulinus* and attack instead species of *Planorbis*. Leiper brought out this selection of hosts as well as the anatomical points of differentiation. This species of schistosome gives off eggs from the capillaries of the intestines forming papillomatous and ulcerating lesions. In the rectum polypi may form so that the condition may be confused with haemorrhoids. The mucus and blood found with the stool may lead to confusion with dysentery. The ova may be carried with the portal current and lodging in the interlobular veins give rise to cirrhosis of the liver.

Schistosoma japonicum.—This is the blood fluke of Oriental countries. It is a common infection in certain parts of China and Japan. It is a rare disease in the Philippines. The male of this parasite has a smooth cuticle and its sides infold more markedly than do those of the other species. There are from 6 to 8 testes. In both sexes the gutbranches reunite in about the lower fourth of the body. This species is characterized by eggs devoid of spines although there is in about 75% a knob-like thickening at one end of the oval egg. This egg is smaller than those of the other species—85 × 60μ as against eggs varying from 125 to 150μ in length for the other species. The eggs of the *S. japonicum* are readily found in the faeces;

they are oval, transparent, and with a smooth shell, within which can be made out the outlines of an embryo. Upon adding water the ciliated embryo begins to show movement in about ten minutes and shortly afterward bursts out of the shell and swims about actively. It is more melon-shaped than the miracidium of *S. haematobium*. Katsurada, by experiments with a cat and dog, has proved that infection will take place through the shaved skin of an animal held in infected water—none of the water being allowed to enter by mouth. Leiper found cercariae showing the absence of a pharynx (characteristic of the genus) in a Japanese mollusk. Such mollusks were teased out in water and laboratory-bred mice immersed therein. One of these mice was killed a month later and adult schistosomes were found in the portal vessels.

According to Bartsch the known mollusk hosts of *S. japonicum* belong to two genera, namely, *Katayama* and *Oncomelania*. The former typified by *K. nosophora* (Robson), Island of Hondo, Japan. Subspecifically distinct from this, he recognizes, *K. nosophora yoshidai*, Island of Kiushiu, Japan and *K. formosana*, Island of Formosa which was described some time ago by Pilsbury and Hirasi as *Blanfordia nosophora*. In China *K. fausti* and *K. fausti cantoni* have been incriminated.

The genus *Oncomelania* acts as the mollusk host in the Yangtze Valley. The miracidia, which break out of the eggs passed with the faeces, when reaching the body of water containing these snails, penetrate the snail and form a sporocyst which shows cercariae by the second week. Mice infected with these cercariae showed parasites in the liver after three weeks. The cercariae, after reaching the lungs, bore through the diaphragm and thus reach the liver. This parasite tends particularly to cause cirrhosis of the liver. In the Japanese infection the symptoms point more to liver and spleen, there beng ascites, cachexia, urticarial spots and some bronchial trouble (urticarial fever). The eggs should be searched for in the mucus cap on the faeces.

Laboratory Diagnosis.—With the vesical bilharziasis we find the terminal-spined egg in the urine. Exceedingly rarely, after profuse haematuria, adult worms may be found in the urinary sediment. It must be remembered that the terminal-spined eggs of *S. haematobium* also may be found in the faeces. The lateral-spined eggs of *S. mansoni* are found in the faeces as is also the case with the eggs of *S. japonicum*. A marked eosinophilia is a feature of the early stages of *S. japonicum* infection and Lawton has noted a similar feature for *S. mansoni* cases. Favorable reports have been made as to the value of complement fixation tests for bilharzial infections, using extracts from infected snails for antigen.

Prophylaxis.—Egg-containing material should be treated to kill the eggs or prevented from obtaining access to water containing snails. Filtering or impounding the cercariae-containing water makes it safe for bathing or drinking.

Cestode or Tape-worm Infections

The cestodes and trematodes constitute the two great divisions of the flat worms. Anatomically, a tape-worm may be considered as a series of individual flukes united in one ribbon-like colony. The cestode segments, or proglottides, are covered by an elastic cuticle and in their interior usually contain striated elliptical bodies composed of calcium carbonate, in size about 5 to 25μ according to the species in which they are found.

These calcareous bodies are characteristic of cestode tissue. They have been mistaken for coccidia. There is no mouth or alimentary canal in tape-worms, the segments absorbing their nourishment through the general surface.

A tape-worm is divided into the segment-producing controlling head and the series of segments or proglottides, together known as the strobila. The head and neck together form the scolex.

The head contains the central nervous tissue and the commencement of the water-vascular system.

Tape-worm heads are provided with suctorial or hook-like organs, or both, to enable them to hold on to the intestinal mucosa.

The hooks when present on the anterior extremity of the head are carried by a protrusible structure called the rostellum.

The importance of the head is generally recognized by the well known fact that the permanent evacuation of one of these parasites is accomplished only when the head as well as the segments is expelled. Otherwise, additional segments will be produced.

Even in tape-worms 25 to 30 feet in length, the head is no larger than a small shot. It carries the suckers or hooklets which best enable us to differentiate the different species. The segments adjacent to the head are immature—the sexually mature ones being found from the middle of the body onward.

T. saginata has about 2000 segments, *T. solium* less than 1000 while *Echinococcus* has only three or four. The sexually mature segment possesses a varying number of testicles; three in *Hymenolepis nana* and as many as 2000 in *Taenia saginata*. As with the flukes, they also have vasa deferentia, cirrus, ovaries, yolk glands, uterus, genital pore, etc. The location of the genital pore and the character of the branching of the uterus are of the greatest importance in differentiation. The mature proglottides are filled with ova. When a special birthpore is present as in Diphyllobothriidae the individual eggs are continuously found in the faeces. When a special birthpore is not present, as in the Taeniidae, eggs are not found in the stool except after rupture of the last segment with subsequent discharge of eggs in the intestinal contents. It is an important practical point that the faeces of a patient with *T. solium* or *T. saginata* may not show any ova, these passing out in the intact segments.

The "hexacanth" or six-hooked embryophore is the essential part of the egg. The embryonic envelope is dissolved off in the alimentary canal of the animal

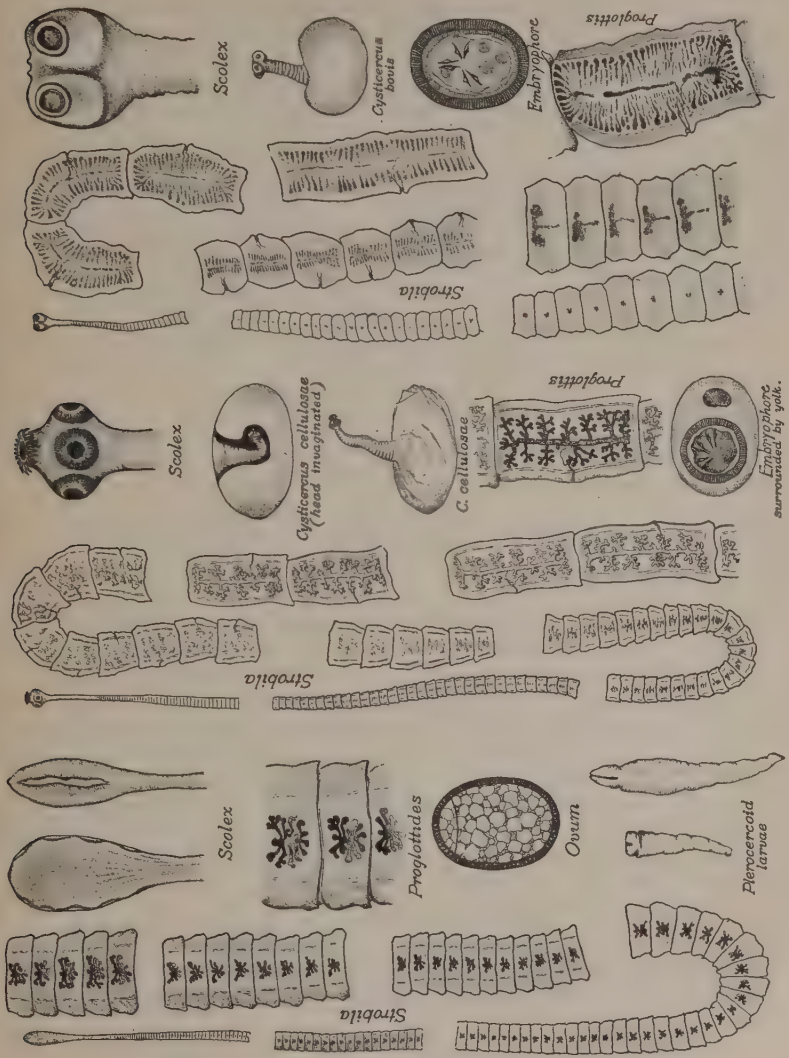


FIG. 128.—Adult and larval stages of cestoda of man.

ingesting it, and the six-hooked embryo bores its way through the gut later to become encysted in various tissues. When the six-hooked embryo reaches its proper tissue, the hooklets are discarded and a scolex similar to the parent one is developed.

At this time we have a bladder-like structure with the scolex inverted in it. This is termed the proscolex stage, *cysticercus*. This little cyst with its scolex when ingested by another animal is digested, and the scolex, establishing itself in the intestine, develops a series of segments. In the Diphyllobothriidae the oval operculated eggs at time of deposit do not show the six-hooked embryo. During the course of several weeks a ciliated embryo, the onchosphere, showing six hooklets is developed and liberated in the water. The procercoid stage is passed in species of *Cyclops* and the plerocercoid stage in species of fresh water fish. During these stages cysts are not formed, but instead solid wormlike creatures.

If the larval stage shows a single cyst and a single head, it is termed *Cysticercus;* if the single cyst shows several heads the term *Coenurus* is applied (does not occur in the tapeworms of man), while with multiple cysts and multiple heads in each cyst the term *Echinococcus* is used.

Where there is very little fluid in the cyst and the larva is of minute size, as with the *Hymenolepis*, the term *Cercocystis* is employed.

Key to Cestode Genera Found in Man

I. Head with two elongated slit-like suckers—Genital pores ventral—Rosette uterus. Bothriocephaloidea.
  (*A*) Single set of genital organs in each segment. *Diphyllobothrium.*
  (*B*) Double set of genital organs in each segment. *Diplogonoporus.*
  (*C*) Immature forms showing characteristics of Bothriocephaloidea—(collective group). *Sparganum.*

II. Head with four cup-like suckers; genital pores lateral. Taenioidea.
  (*A*) Uterus with median stem and a varying number of lateral branches. *Taenia* and *Echinococcus.*
  (*B*) Uterus without median stem and lateral branches.
    (1) Genital pores single. Rostellum with not more than two rows of hooks.
      (*a*) Suckers armed with numerous small hooklets. Fifteen to twenty testicles in each segment. *Davainea.*
      (*b*) Suckers not armed. Three testicles in each segment. *Hymenolepis.*
    (2) Genital pores double. Rostellum with four or five rows of hooks. *Dipylidium.*

Taenioidea Infections

Taenia saginata.—This very widely distributed tape-worm is often termed the unarmed tape-worm, to distinguish it from the *T. solium* or armed tape-worm.

The armed rostellum of *T. solium* is lacking in *T. saginata*, its site being represented by a depression; but in compensation, the suckers of *T. saginata* are much the more powerful. *T. saginata* is from 10 to 25 feet long and has several hundred proglottides. The small pear-shaped head (1/15 inch—1.5 mm.) has four pigmented

elliptical suckers and no hooklets. The segments are plumper than those of *T. solium*, hence the name *saginata*. The single lateral genital pore projects markedly and in a series of segments presents, as a rule, first on one side, and then on the opposite side of the next segment (alternating). The best way to distinguish a segment of the *T. saginata* from the *T. solium* is by counting the number of lateral uterine branches. Those of *T. saginata*, number fifteen to thirty, are quite delicate and branch dichotomously. The lateral divisions of the uterus of the *T. solium* are tree-like in the branching and number only five to twelve on each side.

T. solium has three ovaries while *T. saginata* has only two. The ox is the intermediate host of *T. saginata*. The eggs of *Taenia* have an oval outer shell which is filled with rather translucent, refractile yolk, often in globules. Within the oval shell is the more rounded cell of the six-hooked embryophore with its thick striated membrane. The outer shell is often absent in the eggs found in the faeces, only the shell of the six-hooked embryo being found. The six-hooked embryo, having worked its way from the alimentary canal to the muscles or liver of the ox, becomes encysted (*Cysticercus bovis*). This little bladder-like structure is about ¼ by ⅓ inch, and contains but a small amount of fluid.

The evaginated head does not show hooklets, thus differing from the armed rostellum of the scolex of *Cysticercus cellulosae*.

Man having ingested the cysticercus contained in raw or imperfectly cooked meat, the adult stage becomes established in his alimentary canal in about two months. *Cysticercus bovis* is more abundant in the tongue of cattle than elsewhere in the musculature.

It is probable that the various raw-meat cures have made the infection more common. For this reason it would seem advisable to use other raw meat than beef in such cures.

In Abyssinia the infection is said to be universal and a man without a tape-worm to be a freak. An important point is the fact that the larval stage seldom appears in man. It is this fact which makes it a so much less dangerous parasite than the *T. solium*, which readily establishes a larval existence in man if the ova are introduced into the human stomach. Cooking meat always destroys the cysticercus. A period of about two months elapses after the ingestion of the cysticercus before the mature segments pass out of the rectum. These not only make their exit with the faeces, but are also capable of wandering out at other times. In this they differ from the segments of *T. solium*. *T. saginata* next to *Hymenolepis nana* is the common tape-worm of the United States. Stiles examined several hundred tape-worms in the United States and found only one *T. solium*. In Paris Blanchard found 1000 *T. saginata* to 21 *T. solium*. Certain German statistics, however, show about one-half as many *T. solium* as *T. saginata*.

Abnormalities of the scolex and proglottides are not uncommon with *T. saginata*. This is less frequently the case with *T. solium*.

Taenia solium.—The "measly pork" tape-worm is smaller than the *T. saginata* and differs from it in having a globular head (1/25 inch—1 mm.), with a rostellum which is crowned by 26 to 28 hooklets.

The segments have only five to ten coarse branches and are expelled only at the time of defaecation. The segments or the ova having been ingested by a hog, the six-hooked embryo is liberated and becomes encysted chiefly in the tongue, neck

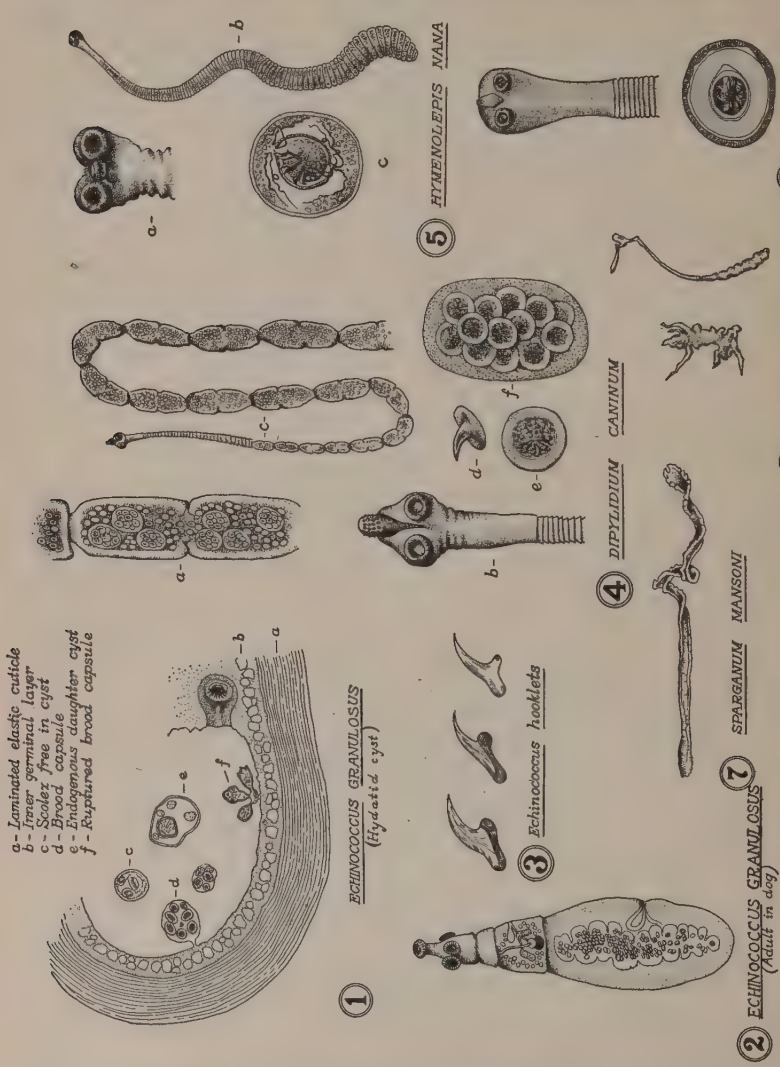


Fig. 129.—Other cestodes of man.

and shoulder muscles of the hog, as an invaginated scolex. Pork containing the cysticercus (*Cysticercus cellulosae*) is known as measly pork. This cysticercus contains much more fluid than that of the ox and is from ¼ to ⅘ inch long. If one

by chance should carry the egg on his fingers to his mouth, as the result of examining mature segments, the larval stage may be established in man. If this infection is not heavy, very few symptoms may be observed. The cysticercus, however, tends to invade the brain, next in frequency the eye, and so causes convulsions, death or blindness. Instead of being only the size of a pea, these cysts, when forming in the brain, may be the size of a walnut or larger. *T. solium* is comparatively common in north Germany, but is exceedingly rare in England and the United States.

Taenia africana.—This is an unarmed tape-worm, only about 5 feet long. It was found in a native soldier in German East Africa.

Garrison has reported from the Philippines a tape-worm with an unarmed rostellum, V-shaped and spiral formation of the uterine stem with compact structure of the gravid uterus under the name of *Taenia philippina*. Another tape-worm, *T. confusa*, of which only segments were found, was reported by Ward from Nebraska.

Hymenolepis nana (Taenia nana).—This is generally known as the dwarf tape-worm—it is the smallest of the human tape-worms. It is from ¼ inch to ½ inch in length, and is less than 1/25 inch in breadth. (10 × 1 mm.).

The genus *Hymenolepis* has lateral genital pores, all of which are on the same side. These lateral genital pores cannot be made out in specimens as ordinarily examined. The head has four suckers and a rostellum, which is usually invaginated. The rostellum has a single row of 24 to 30 hooklets encircling it. Of the 150 to 200 narrow segments the terminal ones are packed with eggs which in the last two or three seem to fill entirely the disintegrating segments. It would seem that the fully mature segments disintegrate and in this way the eggs are set free in the surrounding intestinal contents.

The worms as found in fresh faeces after taeniacidal treatment are frequently in an advanced state of disintegration so that it is impossible to make out the head or hooklets.

The eggs of this species are quite characteristic, there being two distinct membranes. The inner one has two distinct knobs, from which thread-like filaments proceed. The eggs of the *H. diminuta* have a thicker, striated, outer membrane and there are no filaments. The eggs of the *Dipylidium caninum* are similar, but are found in the faeces in aggregations—several eggs in a packet.

The dwarf tape-worm has been found to be the most common tape-worm in the United States. Stiles found it in about 5% of children in a Washington orphanage.

It has been estimated that in certain parts of Italy 10% of the children may be infected. The symptoms, especially nervous ones, may be marked in this infection. It has been incriminated as a cause of chyluria. Although very small, yet the number of parasites may be very great, even more than 1000. In a case that I treated with thymol there were 1500 worms expelled.

H. nana of man has been shown to have a direct development without a separate intermediate host. *H. nana fraterna* (*H. murina*) a parasite of rats was considered by Grassi, Ransom and others as identical to *H. nana*. Braun, Joyeux and others

doubt this identity. However, Woodland (1924) transmitted the parasite of man to seven mice out of thirty to which he fed ova.

If the form in rats is identical with *H. nana*, man probably receives at least part of his infection from food soiled by the excrements of rats and mice.

The life history as given by Grassi for *H. nana fraterna* is that the eggs are ingested and reaching the lower part of the ileum the six-hooked embryo bores into a villus and is transformed into a cercocystis (larva of small dimensions with but little fluid). In about four days the cercocystis has developed a rostellum with hooklets and then leaves the villus to attach itself to the epithelial lining of the villus. In about 12 days the segments are formed and by the end of a month eggs are given off. No intermediate host is required. It seems necessary that the eggs be acted on by the gastric juice as otherwise they will not liberate the embryophore. It is usually considered that the life history of *H. nana* is similar.

H. diminuta is much larger than *H. nana*, varying from 8 to 20 inches in length and about ⅛ inch wide. The suckers are small and the rostellum insignificant and unarmed. It was formerly thought that the intermediate host was a moth; it is now known to be the rat flea. The definitive host is the rat. As man is not liable to eat rat fleas, the infection is rare in man. Twelve cases have been reported for man of which five were from the U. S.

Drepanidotaenia lanceolata (**H. lanceolata**) is common in geese and ducks. It has been reported as occurring once in a boy. This parasite is from 1 to 5 inches long and about ¼ to ½ inch wide. It has a small globular head with a rostellum bearing 8 hooks. The neck is very short.

Dipylidium caninum.—This is a common parasite of dogs and cats. The larval stage is passed in lice and fleas. The cases of human infection have been principally in children, probably from getting dog lice or fleas in their mouths. The number of infections reported for man is about 40 and of these about 30 in children. The head has four suckers and a rostellum, which has three or four rows of encircling hooklets. The segments have the shape of melon seeds and have bilateral genital pores.

Davainea madagascariensis.—It is about 10 inches long. The head has four suckers and a rostellum with 90 hooklets. The suckers have rings of hooklets. The genital pores are unilateral. The cockroach is supposed to be the intermediate host.

There have been about 10 cases reported (Madagascar, Siam and British Guiana). There has been reported a *D. asiatica*, the single specimen, however, lacking a head so that the exact genus is doubtful. It has been reported twice in children in Breslau. It has been suggested that the cockroach may be the intermediate host. Garrison reported cases from the Philippines. *D. formosana* has likewise been reported from Formosa.

Bothriocephaloidea Infections

Diphyllobothrium latum (Dibothriocephalus latus).—This is frequently termed the broad Russian tape-worm. It has a small olive-shaped head (1/10 inch—2.3 mm.) with two deep winding suctorial grooves on each side; it has neither rostellum nor hooklets.

The segments are quite broad, being about ½ by ⅕ inch. At the end of the strobila they are more nearly square. The segments are very numerous, 3000 or more. The fully developed worm is about 30 feet long. The uterus in each segment is rosette-shaped and the genital pore is ventrally situated. The eggs of this species have an operculum and develop a ciliated embryo, the onchosphere. The ciliated embryo swims around and is swallowed by species of *Cyclops* (*C. strenuus; Diaptomus gracilis*). The ciliated covering is lost, the embryo pierces the gut wall, comes to rest underneath the peritoneal covering and in about 15 days develops into the pro-

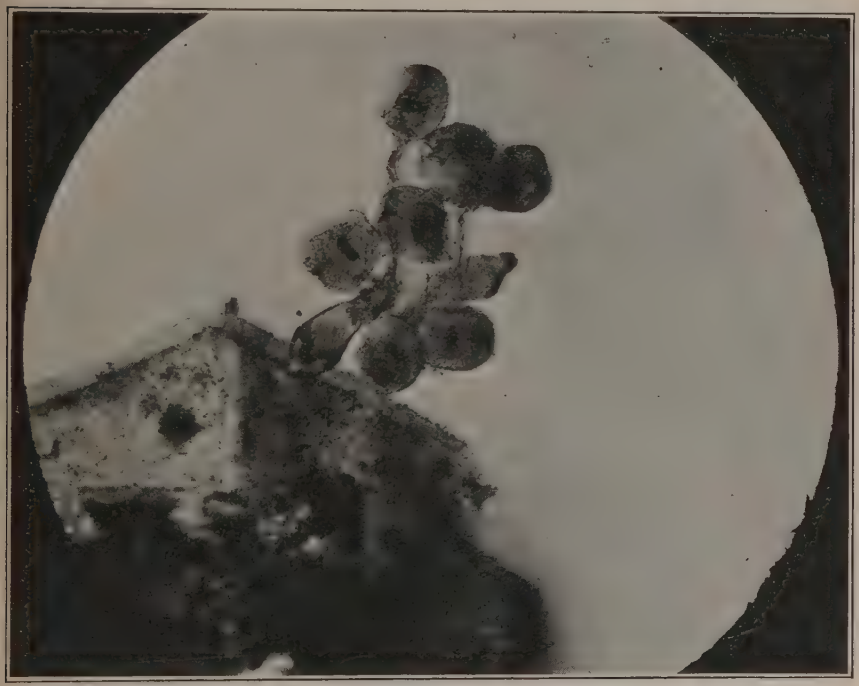


FIG. 130.—Echinococcus cyst wall with a ruptured blood capsule showing scolices. (*From U. S. Naval Medical Bulletin.*)

cercoid larva. When the crustacean is ingested by the second host (some species of fresh water fish) the larva bores through the stomach to make its way to the stomach wall, liver, ovarian mass or the muscles where it becomes encysted as a plerocercoid larva, a worm-like organism with suctorial grooves at its anterior extremity. It varies in length from ⅓ to ⅔ inch. It is said that salting, smoking, or other ordinary methods of preserving fish will not kill it. When swallowed by man, dog or cat, it settles in the small intestine and grows rapidly. In eighteen days it is two feet long and eggs appear in the faeces. This parasite produces intense anaemia similar to pernicious anaemia. It is a frequent parasite in Switzerland, Bavaria, Japan,

Scandinavia, and Russia. Recently several cases have been reported from Northwest U. S. and some of the fish of the waters of that region are said to be infected.

A tape-worm, *Diplogonoporus grandis* has been reported from Japan. In this there are two complete sets of genital organs to each segment.

Somatic Taeniasis

While rarely we may have the larval stage of *T. solium* present in man, and while certain bothriocephalid larvae (*Sparganum mansoni* and *Sparganum proliferum*) infect man, yet they are unimportant as compared with the larval stage of the *Echinococcus granulosus*.

Echinococcus granulosus.—The adult stage of this parasite is passed in the upper small intestines of dogs. It is one of the smallest tape-worms known, being only about $\frac{1}{6}$ inch long. It has a head with four suckers and a rostellum encircled with two rows of hooks. There are only three to four segments. The larval stage, on the contrary, gives one of the largest of larval cestodes. In man it may reach the size of a child's head.

Man contracts the infection from association with dogs. The increase in this serious infection is probably due to the ingestion by dogs of infected parts of hogs and sheep at abattoirs. The disease is peculiarly prevalent in Iceland, 1 in 43 inhabitants being infected. It is also quite common in Australia. As stated above, the adult stage is passed in the intestine of the dog. Should the egg-bearing segments passed by the dog contaminate the hands of man and a single egg be ingested, we may have hundreds of *Echinococcus* larvae produced. The six-hooked embryo, leaving its shell, bores its way through the walls of the alimentary tract and especially seeks the liver, just as the embryo of *T. solium* seeks the brain and eye. The course would seem to be by way of the portal circulation and not by lymphatic channels.

Griffith notes that in Australia from 10 to 15% of hydatid cysts occur in the lungs. The cyst wall is quite thin. Hydatid cachexia seems to appear earlier in lung cases than in liver cases.

While probably 60% of cases have the cysts in the liver yet the kidneys, spleen, omentum, heart and brain may also contain them. Usually only one organ is invaded.

In the development of the cyst, after the embryo has come to rest at some point in the liver, we have formed at first an indistinctly laminated external envelope with coarsely granular fluid contents. Later on the contents become transparent, and two distinct layers can be observed: (1) The external, markedly laminated one, and (2) the internal one, made up of numerous nuclei, embedded in a protoplasmic matrix, and some calcareous corpuscles. When the external layer is incised it curls up by reason of its elasticity. This is characteristic of such a cyst. In addition, we have an enveloping connective tissue capsule formed by the surrounding liver substance.

From the internal lining membrane, known as the parenchymatous or germinal layer, arise various budding processes. These become vesicular and do not have

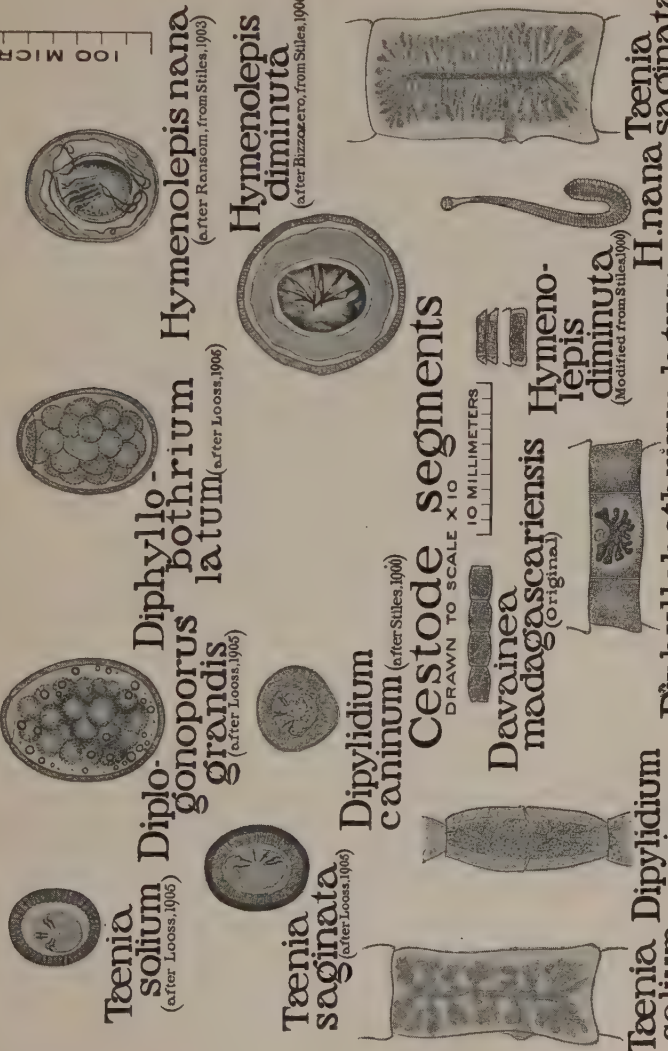


FIG. 131.—Cestode ova.

the external laminated layer. They are called brood capsules and by a process of invagination give rise to a varying number of scolices. It is interesting to note that one egg may develop thousands of scolices. The scolex is about 175μ in diam-

eter, has suckers and two rows of hooklets and is borne on a pedicle. From the parenchymatous layer of the mother cyst, daughter cysts are formed; these have an external stratified layer and an internal parenchymatous one; within them a varying number of brood capsules and scolices may develop. From these daughter cysts, granddaughter cysts may arise—all within the mother cyst—and hence are termed endogenous. At times the daughter cysts work their way external to the mother cyst and proceed to develop in a manner similar to the endogenous formation. The exogenous development is rare in man, but common in hogs.

Hydatids containing no scolices are called sterile. These cysts may be as large as a child's head, but are usually smaller. The fluid of these cysts contains about 1% of NaCl, also a trace of sugar; in addition there is a toxin which produces urticaria and acts as a cardiac depressant. If any quantity should escape into the peritoneal cavity at operation, it may cause death. Aspiration of hydatid cysts is condemned; we must either remove the cyst or stitch the opened cyst to the abdominal walls allowing the cyst to close by granulation (marsupialisation). Hydatids develop very slowly, and the duration of the disease is usually from two to eight years.

Echinococcus multilocularis is possibly due to a species different from *E. granulosus*. In this we have a honeycomb arrangement with cavities filled with a gelatinous material. The majority of these cysts are without scolices. This form of hydatid is very fatal.

Sparganum mansoni (Bothriocephalus liguloides).—This is the larval stage of *Diphyllobothrium mansoni* of dogs and is about 5 to 10 inches long and has been reported 10 times in Japan. It has been found in various parts of the body, as in pleural cavity, tissues about kidney, and in abscess of the thigh. They have been found in the urethra and under the conjunctiva. They resemble ribbon-like strings of fat. The anterior end is thickened and has two suctorial grooves. Yoshida fed a puppy with a larval worm about 8 inches long and enclosed in capsule, which had been removed from the abdominal wall of a woman patient. The adult worm, *D. mansoni*, which developed in the dog resembled closely *D. latum*. The eggs of this species were much longer and narrower than those of *D. latum*.

Sparganum proliferum (Plerocercoides proliferum).—This has been reported from Japan as a larval form in the subcutaneous tissue. Stiles has found these larval forms in skin lesions in Florida. They show themselves as bizarre grub-like forms but may also appear as thread-like bodies. They reproduce by budding. When these buds are detached they give rise to a new worm so that instead of one worm in the subcutaneous cysts we may find several. Adult stage and life history unknown.

CHAPTER XIX

THE ROUND WORMS

CLASSIFICATION OF THE NEMATHELMINTHES (ROUND WORMS)

Class Nematoda

Superfamily	Family	Genus	Species
Rhabdiasoidea	Rhabdiasidae	Strongyloides	S. stercoralis
Trichuroidea	Trichuridae	Trichuris	T. trichiura
	Trichinellidae	Trichinella	T. spiralis
Strongyloidea	Ancylostomidae	Ancylostoma	A. duodenale
			A. braziliense
		Necator	N. americanus
	Strongylidae	Ternidens	T. deminutus
		Oesophagostomum	O. brumpti
			O. thomasi
	Trichostrongylidae	Trichostrongylus	T. colubriformis
		Haemonchus	H. contortus
	Metastrongylidae	Metastrongylus	M. apri
Dioctophymoidea	Dioctophymidae	Dioctophyme	D. renale
Oxyuroidea	Oxyuridae	Enterobius	E. vermicularis
Ascaroidea	Ascaridae	Ascaris	A. lumbricoides
		Toxocara	T. cati
			T. canis
Spiruroidea	Spiruridae	Physaloptera	P. caucasica
Filarioidea	Filariidae	Loa	L. loa
		Wuchereria	W. bancrofti
		Acanthocheilonema	A. perstans
		Onchocerca	O. volvulus
	Dracunculidae	Dracunculus	D. medinensis
Acanthocephala (Class)	Gigantorhynchidae	Macracanthorhynchus	M. hirudinaceus
	Moniliformidae	Moniliformis	M. moniliformis
Annelida (Phylum) Hirudinea (Class)	Hirudinidae	Hirudo	H. medicinalis
		Limnatis	L. nilotica
	Haemadipsidae	Haemadipsa	H. zeylanica

The subphylum Nemathelminthes is divided into two classes: the Nematoda which possess a gut but are without a proboscis and the Acanthocephala in which the gut is absent but proboscis present. The former includes the subclasses Eunematoda and Gordiacea of which the latter are accidentally parasitic in man. The Eunematoda contains normally free-living forms (order Vagantia of some authors)

which are occasionally introduced accidentally into man and the more important human parasitic forms (order Parasita of some authors). At least eight superfamilies of the parasitic forms are recognized.

Note:—The Annelida are grouped with the round worm table for convenience only and not to indicate taxonomic relationship.

Round Worms or Nematodes

All nematodes are covered by a cuticle which varies in thickness, and is frequently ringed. The cuticle is moulted three or four times.

The cuticle is formed by the underlying ectoderm which is, as a rule, markedly developed in four ridges which divide the body into quadrants. Within the ectoderm is the body cavity, a space in which the reproductive organs lie in a clear fluid. The excretory system usually consists of two tubes which discharge near the head.

While the alimentary canal is more or less tube-like in appearance it shows near the mouth a distinct oesophagus which may be non-muscular and of the single row of cells type (Trichuroidea); muscular without globular bulb (Strongyloidea) or muscular with posterior bulb (Oxyuroidea). In Filarioidea and Spiruroidea the oesophagus may be divided tandem. There is a nerve ring around the oesophagus.

The testis and ovary are generally tube-like. The sexes are, as a rule, separate. The male can usually be recognized by its smaller size, its curved or curled posterior end, at times exhibiting an umbrella-like expansion—the copulatory bursa. The spicules, chitinous copulatory structures, may be observed drawn up in the worm or projected out of the cloaca. The genital opening of the female is ventral and usually about the mid-point; that of the male is close to the anus. Certain papillae in the region of the anus are valuable in differentiation.

As a rule, nematodes develop in damp earth from the eggs as rhabditiform larvae. Very few nematodes are viviparous (*Wuchereria, Trichinella*) being usually oviparous (*Ascaris*) less frequently ovoviviparous (*Enterobius*).

The families Gnathostomidae and Anguillulidae are of relatively little importance in human parasitology. Seven cases of human gnathostomiasis mainly due to the *G. spinigerum* have been reported. The family Anguillulidae (normally free-living Eunematoda introduced accidentally in man) contains the genera *Rhabditis* and *Anguillula*. Several species of *Rhabditis* have been reported for man.

A case of infection with a small nematode found in the papules of a skin infection in a French boy is recorded as due to *Rhabditis niellyi*. The present view is that the parasites were embryos of *A. duodenale*, boring into the skin.

Anguillula aceti, the vinegar eel, has been reported from the genito-urinary tract several times. Such cases can be explained by the prior contamination of the urine bottle or by the use on the part of the patient of a vinegar vaginal douche. The nematode larvae found in cases of creeping eruption in Florida by Kirby-Smith, Dove and White and named by them *Agamonematodum migrans* resembles according to Ransom larvae of the superfamily Strongyloidea.

The parasitic Eunematoda are divided into at least eight superfamilies.

KEY TO SUPERFAMILIES

(After Yorke and Maplestone)

1. Heterogenetic, parasitic form parthenogenetic.....Rhabdiasoidea
   Not heterogenetic, parasitic forms sexually differentiated..... 2
2. Oesophagus consisting of a narrow tube running through the centre of a row of single cells for most of its length.....Trichuroidea
   Oesophagus not consisting of a narrow tube running through the center of a row of single cells..... 3
3. Males with a bursa copulatrix..... 4
   Males without a bursa copulatrix..... 5
4. Bursa copulatrix cuticular and supported by rays.....Strongyloidea
   Bursa copulatrix muscular and not supported by rays.....Dioctophymoidea
5. Oesophagus dilated posteriorly into a bulb usually containing a denticular apparatus and frequently separated from the rest of the oesophagus by a constriction.... Oxyuroidea
   Oesophagus not dilated posteriorly into a bulb..... 6
6. Head with three large lobes or lips: relatively stout worms.....Ascaroidea
   Head without three large lobes or lips but with two lateral lips, or 4 or 6 small lips, or lips absent; relatively slender filiform worms..... 7
7. Usually with two lateral lips, chitinous buccal cavity or vestibule usually present, vulva usually in the middle of the body or posterior to it; parasites of alimentary canal, respiratory system, or orbital, nasal or oral cavities..... ....Spiruroidea
   Usually without lips, buccal cavity or vestibule absent or rudimentary, vulva almost invariable in the oesophageal region; parasites of the circulatory or lymphatic system, or muscular, or connective tissue, or of serous cavities...Filarioidea

RHABDIASOIDEA

This superfamily is characterized by having two heterogenetic generations, one of free-living rhabditiform males and females and one of parasitic hermaphroditic or parthenogenetic filariform females. Family Rhabdiasidae.

Rhabdiasidae

Strongyloides stercoralis.—This parasite was formerly thought to be the cause of Cochin-China diarrhoea. It presents two generations: (1) Parasitical or intestinal form, and (2) the free-living or faecal form.

1. The intestinal form (formerly known as *Anguillula intestinalis*) is represented only by females. These are about $\frac{1}{12}$ inch (2 mm.) long and reproduce parthenogenetically. They have a pointed, four-lipped mouth, and a filariform oesophagus

FIG. 132.—A, Egg of *Strongyloides stercoralis* (parasitic mother worm) found in stools of case of chronic diarrhoea; B, Rhabditiform larva from the stools. (*William Sidney Thayer, in Journal of Experimental Medicine.*)

which extends along the anterior fourth of the body. The worm is so translucent that it is difficult to detect it in the jejunal mucosa even with a hand lens. To examine for the worm scrape off the mucosa and search the preparation with a ⅔-inch objective. The anus is situated near the sharpened posterior end, the vulva about the lower third of the body. The double uterus contains a row of eight to ten elliptical eggs which stand out prominently in the posterior part of the body by reason of being almost as wide as the parent worm. The mother worms usually live deep in the mucosa and the embryos emerge from the ova laid in the mucosa. The embryos escape from the eggs while still in the intestine, so that in the faeces we find only actively motile embryos. The eggs, which are strung out in a chain, never appear in the faeces except during purgation. As they greatly resemble hookworm eggs, this is a point of great practical importance. In fresh faeces we find hookworm eggs and *Strongyloides embryos*. The embryos are rather common in stools in the tropics. These embryos have pointed tails and are about 250 × 13μ. They have a double oesophageal bulb. They are about 250μ when they first emerge but may grow until they approximate 500μ in the faeces. If the temperature is low, these rhabditiform embryos develop into filariform embryos, which being ingested form the infecting stage. It has been demonstrated that infection of man may also take place through the skin. If the temperature is high, 25° to 35°C., these embryos develop into:

2. The free-living form (formerly known as *Anguillula stercoralis*). In this we have males and females, with anterior part of oesophagus fusiform and the posterior globular, the male about 1/30 inch (¾ mm.) long with an incurved tail and two spicules and the female about 1/25 inch (1 mm.) long with an attenuated tail; these copulate and we have produced rhabditiform larvae, which later change to filariform ones. At this time the length is about 550 microns. These, being ingested, start up the parasitical generation. If these do not reach the intestine they die out.

Trichuroidea

These have a long thin neck and a thicker terminal portion. The oesophagus is of the nonmuscular single row of cells type. The anus is terminal; there is only one ovary.

The families Trichuridae and Trichinellidae are distinguished by the latter being much smaller, not having a spicule and copulatory sheath and being viviparous.

Trichuridae

Trichuris trichiura (**Trichocephalus dispar**).—This is usually called the whip-worm—the thickened body representing the handle and the narrow neck the lash. It is one of the most common parasites in both temperate and tropical climates.

The egg is very characteristic in having an oval shape with knobs at either extremity. It resembles a platter with handles. The male is almost 2 inches long, and has the terminal portion curled up in a spiral. It has a single terminal spicule.

The female is a little longer than the male, and has the terminal part in the shape of a comma instead of being coiled. The neck contains only the oesophagus which is formed by a groove in large cells lying in a row like a string of pearls. These cells play a digestion rôle. The vulva opens at the upper end of the thickened terminal end which contains an intestine lying between the ovary and uterus. The great powers of resistance of the ova may account for the general distribution of this parasite; they may live for months under conditions of freezing and so forth. There is no intermediate host. The worm arrives at sexual maturity in about one month after ingestion. The whip-worm prefers the caecum, but also lives in the lower end of the ileum and the appendix.

The neck burrows into the mucosa, and much importance has been attributed by the French to the possibility of this paving a way for the entrance of pathogenic bacteria. Whip-worms do not as a rule produce serious symptoms. The patient may harbor parasites in considerable numbers for a long time without inconvenience. Rarely there may be a symptom-complex with severe anaemia, pronounced nervous symptoms and gastrointestinal disturbance.

Trichinellidae

Trichinella spiralis (**Trichina spiralis**).—The cause of trichinosis is usually termed *Trichina spiralis* in medical works.

The adults live in the duodenum and jejunum. They can be seen with an ordinary magnifying glass. There is a gradual thickening of the posterior portion of the body. The males are about $\frac{1}{16}$ inch (1.5 mm.) long with two tongue-like caudal appendages and without a spicule. These two lateral projections enable the male to hold the female in copulation—the cloaca being evaginated to act as a penis

The females are about 1/7 inch (3 to 4 mm) long. The female gives off embryos from the vulva which is near the mouth end (viviparous).

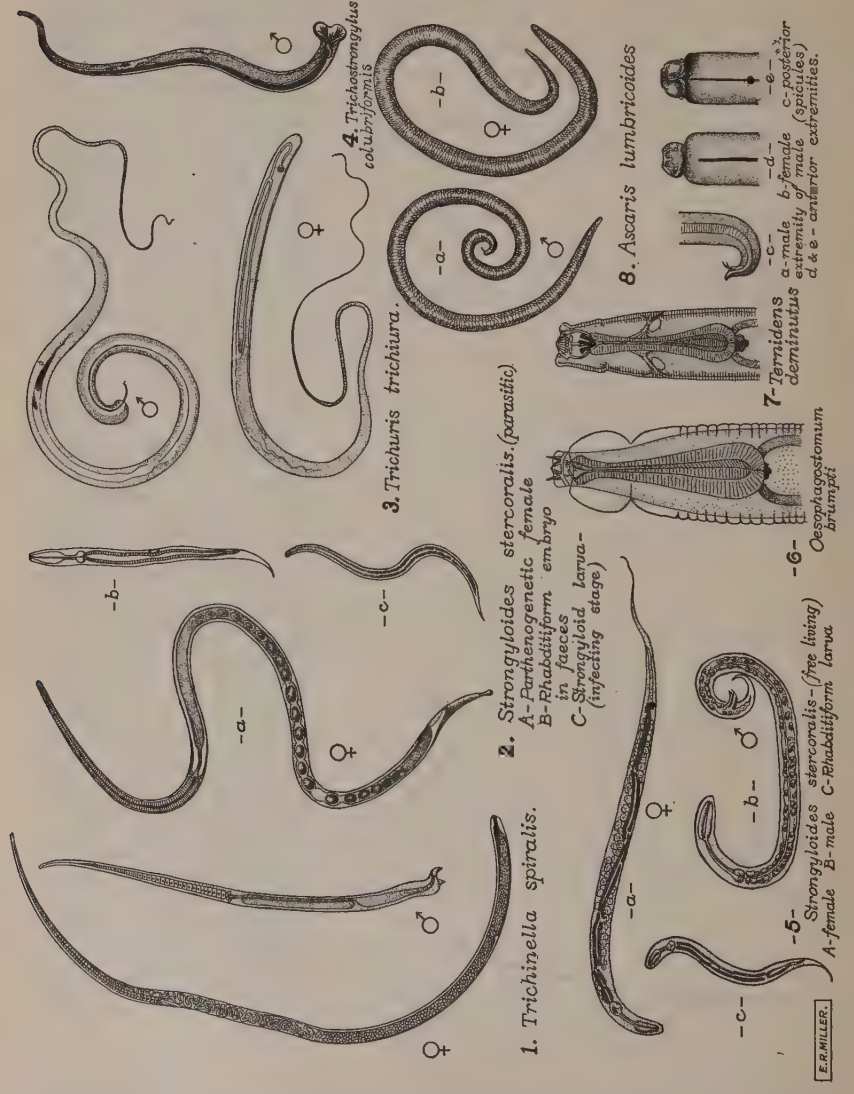


FIG. 133.—Some of the human nematodes.

The male is about 40μ broad and has a prominent testicular enlargement filling the posterior extremity. The female is about 60μ broad and has a rounded posterior extremity with a prominent slit-like cloaca. It is in this posterior extremity that

the female increases in size as she becomes filled with eggs. The vulva is in the anterior third. After fertilization of the females the males die, and the females bore into the intestinal mucosa and begin to produce embryos to the number of more than 1000 each. These gain access to the lymph channels and are distributed by the blood stream to the striated muscles. Embryos reaching other tissues fail to develop.

It is about ten days before they reach their resting place in the muscle. There they become encysted as the oval lemon-shaped areas containing coiled-up embryos with which everyone is familiar. These oval areas are about 450 × 250μ and have a chitinous capsule.

They are found chiefly in the muscle fibers of the tongue and diaphragm.

Encysted trichinae may remain alive as long as ten to twenty years; finally, however, the cyst undergoes calcareous infiltration and the embryo dies.

FIG. 134.—*Trichinella spiralis.* (*Ziegler.*)

When uncoiled the embryo is about 1 mm. long with the mouth at the attenuated end.

Among cannibals it would be easy to keep the cycle going by eating improperly cooked or raw human meat, the parasite being thus transmitted; but this can not explain the transmission among civilized men.

The following is the life history: Man obtains his infection from eating raw pork, the embryos encysted in the muscle of the hog being liberated in the stomach, and the males and females developing in the intestine as above described.

The hog may gain his infection by eating the meat of other hogs or rats. These rats eat scraps of pork at slaughter houses and become infected. Being cannibals, rats when once infected continue to propagate the infection.

In man, during the first two or three days while the adults are breeding in the intestine, we have gastrointestinal symptoms.

It is during this period or at any rate before the fifth day that purging may be of benefit. About ten or twenty days after infection the embryos begin to wander and we have the acute muscle pains. In the diagnosis we should try to obtain specimens

of the pork which has caused the trouble in order to examine for encysted trichinae, or to feed to white rats or rabbits, subsequently examining the diaphragm of these animals for encysted trichinae or the intestine for adult trichinae. Excision of a small piece of the deltoid of man may confirm the diagnosis. The best method during the stage of wandering is to take blood in 3% acetic acid, centrifuge, and examine for larvae.

During the diarrhoeal stage we may examine the stools for adult worms, in particular dead males or possibly actively motile embryos—these latter are about 90 × 6μ.

Always examine the blood for eosinophilia.

It is well to remember that the parts of meat which trichinae prefer (muscle of diaphragm, of neck, etc.) are often used in sausage. Unfortunately it is almost impossible to detect the embryos in sausage meat.

Strongyloidea

In this superfamily the male has a caudal bursa, a prehensile sort of expansion at the posterior end for copulatory purposes.

The mouth is usually provided with six papillae and at times with a chitinous armature. Oesophagus of adult is without posterior globular bulb but may be greatly swollen.

Families discussed: Ancylostomidae in which there is a well developed caudal bursa and buccal capsule. Aperture of buccal capsule guarded by cutting plates or teeth; Strongylidae with well developed caudal bursa and buccal capsule. Aperture of latter guarded by a corona radiata; Trichostrongylidae having a well developed caudal bursa but without buccal capsule or when present rudimentary; Metastrongylidae with a poorly developed caudal bursa having atypical rays and buccal capsule absent or poorly developed.

Ancylostomidae

The hookworm infections of man come almost entirely from two parasites, *Ancylostoma duodenale*, the Old World species, and *Necator americanus*, which is generally called the New World species from its first having been reported from the U. S. by Stiles.

Two other species reported for man *A. braziliense* and *A. ceylanicum* are now generally considered identical.

The male hookworms are a little more than 1/3 inch (9 mm.) long and the female a little more than 1/2 inch (13 mm.) in length.

The males can readily be distinguished by their posterior, umbrella-like expansion or copulatory bursa. The tail of the female is pointed. The vulva of *A. duodenale* is located in lower half of the ventral surface; that of *N. americanus* in upper half.

The large, oval mouth of the Old World hookworm has four claw-like teeth on the ventral side of the buccal cavity and two knob-like teeth on the dorsal aspect. It also has a pair of ventral lancets below the four ventral teeth. One cannot make out a dorso-median tooth. In *N. americanus* the buccal capsule is round, smaller and the ventral

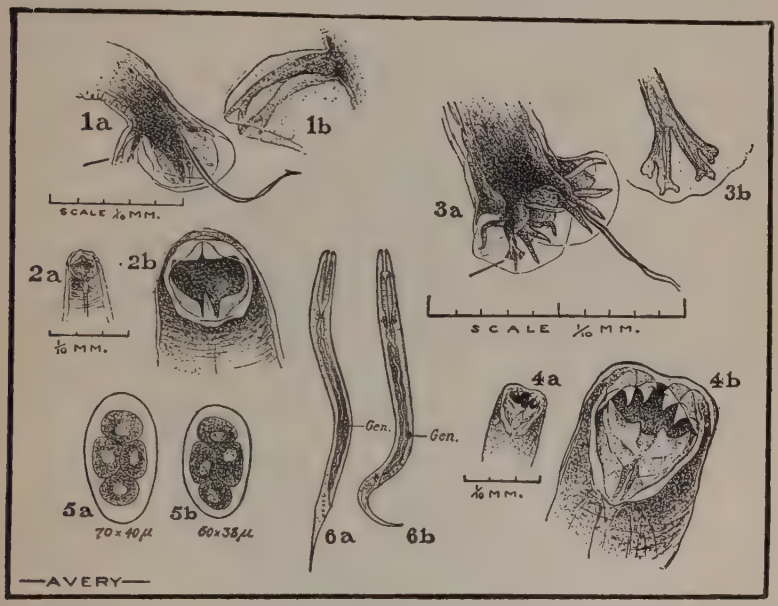


FIG. 135.—1a, Copulatory bursa of *Necator americanus*, showing the deep cleft dividing the branches of the dorsal ray and the bipartite tips of the branches; also showing the fusion of the spicules to terminate in a single barb. Scale 1/10 mm. 1b, Branches of dorsal ray magnified. 2a, The buccal capsule of *N. americanus*. 2b, The sam magnified. 3a, Cop. bursa of *Ancylostoma duodenale*, showing shallow clefts between branches of the dorsal ray and the tridigitate terminations. Spicules hair-like. 3b, The dorsal ray magnified. 4a, The buccal capsule of *A. duodenale*, showing the much larger mouth opening and the prominent hook-like ventral teeth. 4b, The same magnified. 5a, Egg of *N. americanus*. 5b, Egg of *A. duodenale*. 6a, Rhabditiform larva of *Strongyloides* as seen in fresh faeces. 6b. Rhabditiform larva of hookworm in faeces eight to twelve hours after passage of stool.

teeth are replaced by chitinous plates. Dorsally there are two similar but only slightly developed lips or plates. A very prominent, conical dorso-median tooth projects into the buccal cavity. Through it passes the duct of the dorsal oesophageal gland. There are also four buccal lancets.

The copulatory bursa of the *Necator americanus* is also different, being terminally bipartite and deeply cleft in the division of the dorsal ray, rather than tripartite and shallow, as with *A. duodenale*. The two long thin spicules end separately in the latter species whereas in *Necator* they fuse and terminate in a single barb.

The anterior extremity of *Ancylostoma* bends in the same direction as the general body curve while that of *Necator* hooks back in an opposite direction to the body curve. In general, *Ancylostoma* is larger and thicker than *Necator*.

A. braziliense is somewhat smaller than *A. duodenale* and in the copulatory bursa of the male we have a deeper cleft in the dorsal ray and two rather long tips

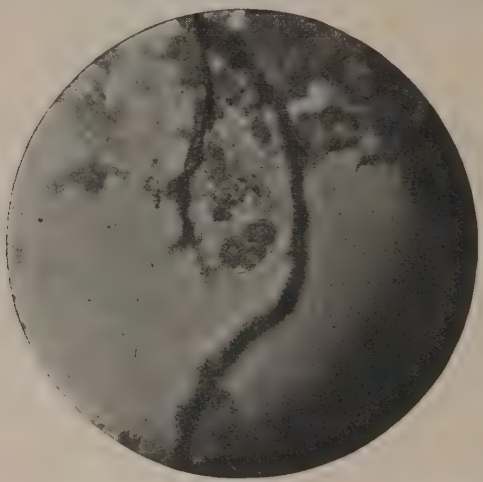


FIG. 136.—Ovum of *Ancylostoma duodenale*. By J. A. Thomson. (*Jeffreys and Maxwell.*)

to each branch instead of the shallow cleft and three stumpy processes of the two branches as in *A. duodenale*. The internal pair of ventral teeth in the former are much smaller.

Goeze found a hookworm in a badger in 1782. He named the parasite *Ascaris criniformis*. Froelich, in 1789, found hookworms in the fox and called them hookworms from the hook-like ribs of the copulatory bursa. He proposed the generic name *Uncinaria*. Therefore *Uncinaria* belongs to the hookworms of the fox and is not valid for any human species.

In 1838, Dubini found a hookworm as a human parasite. On account of the four ventral teeth projecting from the mouth he gave it the name *Agchylostoma* or correctly *Ancylostoma* (αγχυλοσ, hook, and στομα, mouth).

Bilharz and Griesinger noted the connection of the parasite with Egyptian chlorosis, but it was not until the time of the St. Gothard tunnel (1880), that the importance of the parasite was recognized. Grassi noted the diagnostic value of

the ova in faeces in 1878. In 1902, Stiles noted and described the hookworm found in the United States as different and proposed the name *Uncinaria americana*, later changed to *Necator americanus*. A. J. Smith had also recognized the morphological differences.

Hookworms may be found in the small intestine (jejunum) of man in enormous numbers. They produce their effects either by feeding on the mucosa or by causing loss of blood.

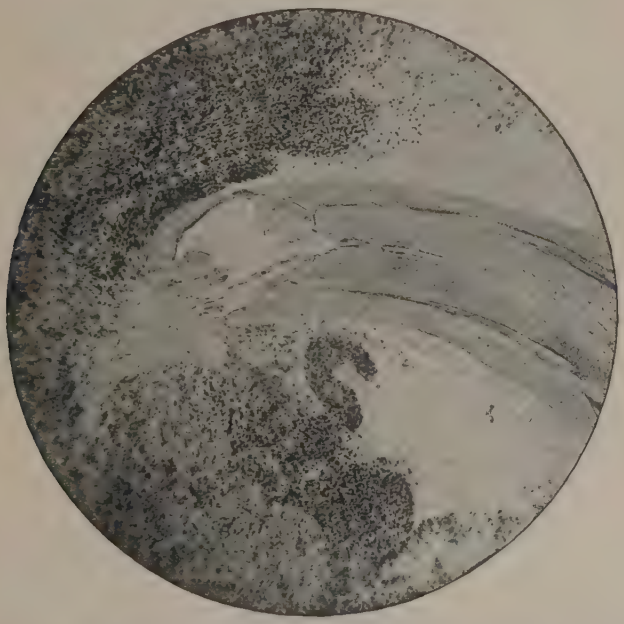


FIG. 137.—Longitudinal section through a hookworm attached to the intestinal mucosa, about six hours after the death of the carrier. A portion of the submucous coat is drawn into the buccal cavity and trails along cord-like into the oesophagus, in which 4 small tissue nuclei are still discernible. Section, 0.01 mm. thick. (*From Mense.*)

Life History.—The delicate-shelled eggs pass out in the faeces, and in one or two days a rhabditiform embryo (200 × 14 microns) is produced.

As a practical point, the anaerobic conditions in the intestines seem to prevent development of the hookworm ova or at any rate the absence of the oxygen, so necessary for the segmentations preliminary to the formation of the embryo, prevents it. Therefore hookworm ova in freshly passed faeces never show other than commencing segmentation while development of the larvae of *Strongyloides* takes place

in the intestines, so that in freshly passed faeces we find, generally, actively moving larvae or at least eggs containing fully developed embryos. Hookworm ova very rarely show more than four segments or exceptionally eight in the freshly passed egg.

In the presence of oxygen these ova rapidly develop into larvae, particularly at a temperature of about 27°C. Beyond 37°C. and below 14°C., development does not seem to take place.

The rhabditiform larvae grow rapidly and by the third day are about 300 microns long and undergo a primary moulting. By the fifth day the bulb-like swellings disappear and the larva becomes possessed of a straight oesophagus, thereby becoming a strongyloid or filariform larva.

It then undergoes a second ecdysis or moulting, but instead of casting off this old covering, it retains it as a protecting sheath (encystment). The larva, now fullgrown, is about 550 × 24μ. Before becoming encysted hookworm larvae are readily destroyed by the action of the sun or chemicals or even by dilution of the faeces, especially with urine. Cort and his coworkers have found that it is common occurrence for mature larvae to lose their sheaths while living in the soil, and to continue their lives in the unsheathed state. The proportion losing their sheaths varies from 52 to 98%. It has been supposed that the larvae can live in this stage for months, provided moisture and shade are present. Cort has found however that under usual tropical conditions hookworm larvae die out quickly, disappearing almost completely from the soil in six weeks. After the second moulting the larva ceases to take food but is actively motile so that it can crawl up blades of grass or vertical sides of mines. Cort states that hookworm larvae, as far as their own activities are concerned, are definitely limited to the place of development, experiments showing that the larvae did not migrate over 4 inches.

This is the infecting stage in which the larvae bore their way into the skin, which is the usual method of infection, or, occasionally, enter the mouth on vegetables or otherwise. The soil in the area of the egg-carrying stool becomes infected with these larvae and persons coming in contact with such soil are exposed to the infection.

Looss thought that they entered the skin by way of the hair follicles but the idea now is that they can bore into any part of the skin. It requires only a few minutes for the larvae to penetrate. From the subcutaneous tissues they effect an entrance into lymphatics or veins, go to the right heart, thence to lungs. From the alveolar capillaries they pass into the pulmonary alveoli, thence up the bronchi and trachea, to pass out of the larynx and then down the oesophagus to the stomach. The larva loses its protecting sheath in the stomach and in a few days develops a provisional buccal capsule.

By the end of the second week, after another ecdysis, the larvae have grown to be about 2 mm. long and 130 microns broad and in about four weeks become adults, usually in the jejunum, where, after fertilization of the females by the males, the giving off of eggs begins. The adults attach themselves to the mucosa of the intestine, feeding on the deeper structures of the mucosa, or on the tissues of the submucosa.

Sambon believes that the larvae can work their way into the jejunum without going there by way of the trachea and oesophagus.

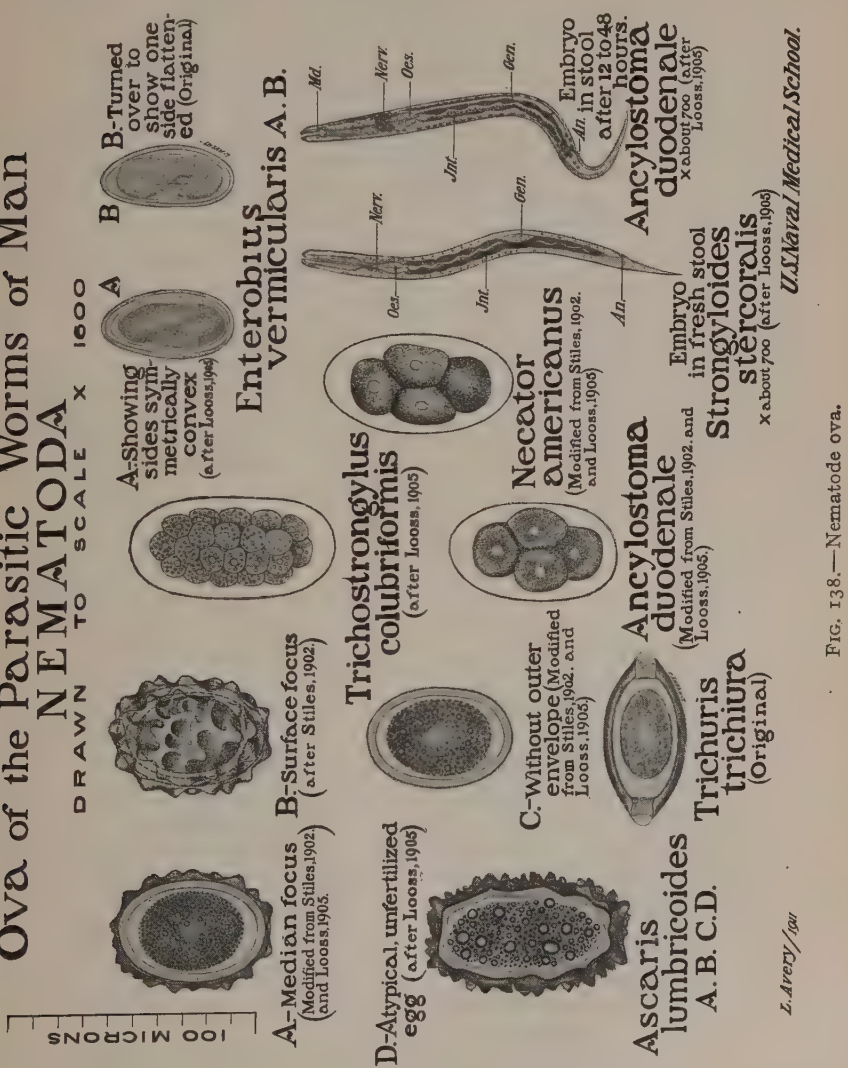


FIG. 138.—Nematode ova.

By providing an exit to the trachea, Fülleborn demonstrated that in dogs, infected with the dog hookworm, great numbers of larvae poured out of the trachea. In other dogs he stitched the oesophagus to the skin and noted larvae coming out of

these openings. In these dogs, with the ordinary channel obstructed, infection did occur with, however, only a few worms, thus showing the truth of Sambon's views but at the same time demonstrating the unimportance of such a route of infection.

The rhabditiform larvae of the hookworm and *Strongyloides* are very similar. In the former however there is a narrow deep mouth capsule, cylindrical in shape, with heavy chitinous walls, shown as 2 bright lines under the microscope. In *Strongyloides* the oral cavity is thin-walled and quite shallow. In the following stage of development the filariform oesophagus of the hookworm is found to be only ⅓ that of the body length, whereas in *Strongyloides* it is one half of the body length. Also the genital anlage of *Strongyloides* is much larger than that of *Ancylostoma*, about 30μ long as against 4 or 5μ for *Ancylostoma*.

Laboratory Diagnosis.—As a matter of fact the diagnosis is almost invariably made by finding hookworm ova in the faeces. The eggs are oval and thin-shelled with a wide, clear, glassy zone separating the more or less segmented, granular central portion from the shell. Formed stools are more satisfactory for examination than the liquid ones resulting from a dose of salts. Put about 2 drops of water or 1% trikresol solution in the center of a glass slide and emulsify in it as much of the faeces as is held by the spatulate end of a wooden toothpick. A small piece of wood or a match stick will answer. These preparations can be readily examined without a cover glass, using a ⅔-inch objective, with a 1-inch ocular.

It is usually stated that about 500 worms must be present for several months to produce symptoms. Grassi has thought that the presence of 150 eggs in 0.01 Gm. faeces indicates the presence of 1000 worms, of which 25% would be males. There may be as many as 4,000,000 eggs in a stool.

Bass has proposed the following method for the examination of faeces for ova. The faeces, which have been made fluid, should be centrifuged and the supernatant fluid containing vegetable débris poured off. The sediment contains hookworm eggs. Then pour on sediment a calcium chloride solution of sp. gr. 1.050. Again centrifuge and decant. Next add calcium chloride solution of a sp. gr. of 1.250 and centrifuge. This brings to the surface the hookworm eggs which may be pipetted off. As a rule, the finding of hookworm eggs is very easy without such a technique.

We have been using Barber's technique. Emulsify the faeces in equal parts of glycerin and saturated salt solution on a slide. The eggs rise to the surface and are easily picked up with the ⅔-inch objective. As a centrifuge method Barber emulsifies faeces in this same mixture which brings the eggs to the surface. A wisp of cotton is placed on the surface and 3 or 4 drops of melted agar dropped on the cotton. The disc of agar is removed with the cotton, deposited on a slide and examined for entangled eggs.

Clayton Lane recommends a technique which he designates as the levitation method. In this procedure the concentrated sediment of a centrifuged specimen is transferred to a glass slide, where it is mixed with 1 cc. of water. The slide is allowed to stand for five minutes and is then immersed in water and manipulated until all coarse matter has floated free. The hookworm ova stick firmly to the

slide and are not washed away. Lane reports that on an average this method results in a ten-fold concentration of ova.

McVail's Method is: Across a glass slide, 2 cm. from each end, draw grease line. Fill space so formed to some depth with a thick emulsion of faeces. Let stand ten minutes. The ova settle and adhere because of sticky capsule.

Lift slide with both hands; immerse gently in a bowl of clear water. Upon removal, faecal matter is washed off leaving ova.

In certain cases, where a microscope is not available, the diagnosis may be made by finding the worms in the stool following treatment.

The presence of eosinophilia is of great assistance in diagnosis but it should be remembered that not rarely severe cases of the disease fail to show any excess of eosinophiles.

Charcot-Leyden crystals are often present in hookworm stools.

It has been claimed that where ordinary microscopical examination for ova will show 40% of infections and methods involving concentration 55% that cultural methods will show 99%. A convenient method of culturing is to make a pile of filter paper circles of 2 inches diameter and about ¼ inch high and place in the center of a 4-inch Petri dish. Fill the dish with water about to the height of the filter paper and spread a thick layer of faeces on the top of the filter paper island. The larvae hatch out in about six days and swim out into the clear surrounding water. They are best found by centrifuging the fluid containing them.

Strongylidae

Ternidens deminutus (Triodontophorus deminutus).—This is a small round worm with three forked teeth taking origin from the

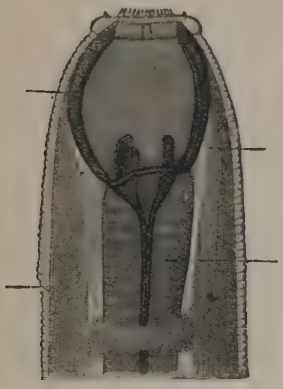


FIG. 139.

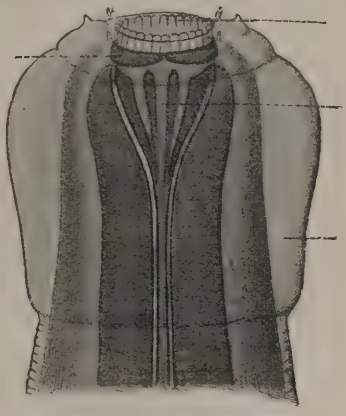


FIG. 140.

FIG. 139.—*Ternidens deminutus.* Head showing mouth capsule surrounded by a crown of leaflets. (*After Leiper from Mense.*)

FIG. 140.—*Oesophagostomum.* Head showing crown with leaflets at the entrance of the mouth capsule. Note bladder-like thickening of the cuticle around the anterior extremity. (*After Raillet and Henry from Mense.*)

pharyngeal lobes. The collar-like mouth orifice is made up of 22 rounded plates just inside the round mouth opening. These parasites are less than ½ inch long and have once been found in the intestinal canal.

Oesophagostomum brumpti.—Six young females were found in a cyst of the colon in an African negro. They were about ⅓ inch (8 mm.) long. The anterior end presents an ovoid protuberance with a second cuticular inflation just below it. This is termed the peristomic collar. The buccal capsule is very shallow and surrounded by about a dozen plates. The mouth has six papillae.

Oes. thomasi.—This species has recently been reported by Thomas in a native of Brazil. It has 38 leaflets in the external crown. The large and small intestines of this case showed numerous cysts, each containing one worm.

Trichostrongylidae

Trichostrongylus colubriformis.—This is a small strongyle formerly known as *Strongylus subtilis*.

The male is about 1/6 inch (4 mm.) long, and the female about ¼ inch (6 mm.). Anteriorly it tapers to a pointed head end which is only about one-tenth the thickness of the posterior extremity. The male has a bursa and two prominent equal spicules. The ova resemble those of hookworm but are larger (73 to 90 × 42μ) and show later stages of segmentation.

It has been found in the upper part of the small intestine of inhabitants of Egypt and Japan. It is a frequent parasite of sheep and goats in the U. S. and may exist in man in regions wherever these animals are common. Stiles, however, during the progress of his Southern States hookworm work, failed to find the infection in man although he kept in mind the possibility of its occurrence. It does not appear to produce symptoms.

Haemonchus contortus.—This is an intestinal parasite of sheep but has been reported for man. Males are about ⅔ inch long (15 mm.) and females about 1 inch long (25 mm.). The anterior end shows two tooth-like papillae directed dorsad.

Metastrongylidae

Metastrongylus apri (Strongylus apri).—This nematode is common in the lungs of hogs, producing a bronchitis in young animals but apparently harmless for adult ones. It has been reported once from the lungs of a six-year-old boy.

The male is about 1 inch (25 mm.) long with two long spicules. The female is about 2 inches long and has a sharply hooked posterior extremity with the vulva just beyond the bend. The mouth has two lips each with three lobes. The eggs contain embryos when laid. It probably does not require an intermediate host.

Dioctophymoidea

Large worms characterized by males having a closed, bell-shaped caudal bursa without rays. Mouth hexagonal with six to eighteen papillae. Oesophagus very long without bulb. Family Dioctophymidae.

Dioctophymidae

Dioctophyme renale (Eustrongylus gigas).—This is the largest round worm infecting man; it is usually found in the pelvis of the kidney (giant strongyle).

Two or more worms may so distend the kidney as to convert it into a mere shell. Pain, haematuria and other symptoms of pyelitis, together with the finding of the eggs, make the diagnosis. There seem to be seven authentic and eight doubtful cases of infection in man. Many of the reported cases were simply fibrinous clots from ureters or wandering round worms.

The females are about 40 inches (1 m.) long and about ⅓ inch (8 mm.) in breadth while the male is about 10 inches (25 cm.) long.

The collar-like copulatory bursa of the male distinguishes it from *Ascaris* as does also the dark-red color. The source of infection is unknown but it has been suggested that the larval stage may exist in fish.

The very characteristic ova, with gouged-out oval depressions, may be found in the urine, and are diagnostically confirmatory.

Oxyuroidea

Worms with three lipped mouth. Lips simple or indistinct. Oesophagus showing definite posterior bulb. Example, pin-worms of family Oxyuridae.

Oxyuridae

Enterobius vermicularis (Oxyuris vermicularis).—This parasite is also known as the pin-worm or seat-worm. It is more frequent in children than in adults.

The male is about ⅙ in. (4 mm.) long and the female a little less than ½ inch (12 mm.) in length. The male has an incurved tail with a single spicule and the female a long tapering tail. The vulva is in the upper third.

These worms have a clear slightly bulbous Turkish pipe mouth-piece-like projection surrounding the three-lipped anterior extremity. There is a well-marked bulb oesophagus.

The eggs are thin-shelled, plano-convex, and show a coiled-up embryo. After ingestion of eggs, the adults develop in the small intestine where copulation takes

place; the males then die. The fertilized females go to the caecum and colon where they remain until they reach maturity. At this time the females wander to the rectum where they either expel their ova or themselves work their way out of the anus. This usually occurs at night, and the scratching induced by the itching causes the eggs to be widely spread about the region of the anus. The worms may also wander into the vagina, urethra, or under the prepuce. It will be seen that as a result of the scratching, the fingers become contaminated with ova which may be carried to the mouth and so cause a fresh infection, no intermediate host being required. The examination of the material under the finger nails of children harboring this parasite may show eggs under the microscope. A knowledge of the life history—the early location in the small intestine, and later on in the large—shows that treatment should be dual in its direction—enemata for the gravid female in the rectum and santonin and calomel for the young adults in the small intestine.

The diagnosis is preferably made by examining the stools for the white, thread-like females which are expelled after a diagnostic dose of calomel and salts, rather than by searching for the eggs.

These females, which are packed with embryo-containing eggs, may be seen wriggling on the surface of the freshly passed faeces. In handling these worms one should be careful as they are apt to cause infection should the eggs get on the fingers.

Oxyuris incognita.—Eggs found in faeces of recruits during the war were considered by Kofoid and White as probably a new species of *Oxyuris*. Later, Sandground, working in Australia identified similar eggs in faeces of man as those of a common parasite of food plants (*Heterodera radicicola*).

Ascaroidea

These have a mouth commonly provided with three prominent lips supplied with papillae, one dorsal and two ventral. Oesophagus muscular and usually without posterior bulb. As a rule an intermediate host is not required. Example, common large intestinal ascarid of family Ascaridae.

Ascaridae

Ascaris lumbricoides.—The male round or eel worm is from 5 to 8 inches (18 cm.) long and the female from 7 to 15 inches (30 cm.) in length. They are grayish to reddish in color and are from 1/7 to 1/4 inch (5 mm.) in diameter.

It is probably the most common parasite of man, especially in children, and as it does not require an intermediate host infection takes place through food or drink or by the fingers in the case of children who have been playing where soil pollution exists. It is found in temperate climates as well as tropical ones and has been reported from Arctic regions.

Stewart noted that *Ascaris* eggs hatch out in the intestines of rats or mice and the larval worms then migrate to liver, spleen and lungs. After undergoing various

developmental stages they leave the lungs, go up the trachea and then down the oesophagus to the intestines. It was considered that these intestinal forms might pass out in the rat's faeces, contaminate food and infect man. Ransom notes the same development in a young pig fed *Ascaris* eggs as for mice, rats and guinea pigs. Stewart has also reported infection of pigs with *A. suilla* ripe eggs, thus demonstrating that an intermediate host was not necessary. Man and the pig being efficient hosts show development to maturity in the intestines which in guinea pigs, mice and rats does not occur. The hog round worm (*A. suilla*) is closely related to the human lumbricoid one. Yoshida swallowed larvae taken from lungs of guinea pigs and 10 weeks later showed eggs in his stools. Ransom stated that the human and pig *Ascaris* are the same species and calls attention to the dangers of becoming infected from hog faeces. He notes that a serious lung trouble in pigs (thumps) is caused by *Ascaris* larvae passing through the lungs and considers that children with heavy infections would show similar trouble. When the larvae hatch out in the intestines they reach the liver through the portal circulation. When first hatched

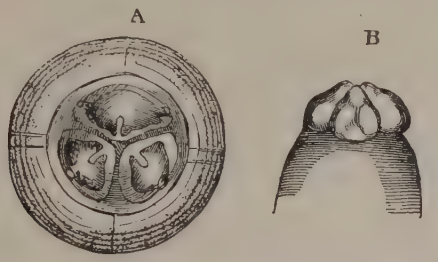


FIG. 141.—Anterior extremity of *Ascaris lumbricoides;* A, seen from front; B, seen from dorsal surface. (*Tyson after Railliet.*)

they are 250 × 12μ but while in the lungs they grow to a length of one or two millimeters before going up the trachea and then down the oesophagus on the way to the intestines. Ransom notes that young pigs suffer more from lung injury than older animals and believes this true of man.

Opposed to Ransom's conclusions as to the identity of *A. lumbricoides* and *suilla* are the results of experiments by Koino. He himself swallowed two thousand mature eggs of *A. lumbricoides*, and developed pronounced symptoms of lung involvement with bloody sputum containing larvae. Fifty days later, following an anthelminthic, 667 worms were recovered. Another person, who at the same time ingested eggs of *A. suilla*, developed mild symptoms, possibly indicative of larval migration, but no larvae were recovered from the sputum nor were worms recovered from the faeces. Stiles considers the *A. lumbricoides* and the *A. suilla* at least physiologically distinct. The Caldwells, as a result of a sanitary survey, state that the epidemiological evidence is against the pig and human *Ascaris* being identical.

The normal habitat is the upper part of the small intestine, hence the ease with which they are vomited up. The three papillae-like lips with a constriction just behind are easily studied with a hand glass. The very long, whitish, convoluted, thread-like tubes of the uterus lead to the opening of the vulva anteriorly and ven-

trally. The male has two large lance-like spicules which project from a subterminal cloaca. The posterior extremity of the male is curved ventrally and has seven pairs of postanal papillae.

The body of the worm is transversely striated and resembles the ordinary earthworm, but is more grayish than red. The ova are very characteristic with a rough mammillated exterior due to an albuminous covering. This at times is shelled off and we have a smooth egg which may be mistaken for eggs of other parasites. The eggs leave the body in the faeces and after a long time—a few weeks to several months, according to temperature—become infective for man. During this time an embryo develops, which remains in the shell until swallowed by man. It is stated that they will remain alive for years. After being swallowed, the embryo leaves the egg, pierces the gut wall and reaches the liver through the portal system, thence the lungs through the venous circulation. It then reaches the intestinal tract in the same manner as the hookworm larvae. Males and females develop in the small intestines. The passage through the bloodstream seems to be essential for the development of the worm.

In countries where such parasites abound, as in Guam and the Philippines, the possibility of their getting into the peritoneal cavity through operative measures on the intestine must always be thought of.

Guiart considers it probable that *Ascaris* may suck blood, produce intestinal ulcerations and bacterial infections, and perforate intestine. Their entrance into bile ducts or into larynx (vomited) must be considered.

At autopsy they may be found perforating the appendix or even filling up the pancreatic duct.

Some think that the symptoms of itching of nose and anus, vertigo, or convulsions and anaemia, which may accompany infection, may be due to a toxin secreted by the worm.

Toxocara cati and *T. canis* the common ascarids of the cat and dog respectively have been reported for man. They are much smaller than *A. lumbricoides* averaging about three inches in length and are characterized by wing-like projections from the anterior end by reason of which they are called the arrow-headed ascarids. The eggs are thick shelled and somewhat similar to those of *A. lumbricoides*. The classification of the arrow-headed ascarids is at present somewhat confused, some authors preferring the generic names *Belascaris* and *Toxascaris*.

Other Ascaridae reported from man are *A. texana* and *A. maritima*, only one case each.

Spiruroidea

Members of this superfamily may be plump resembling *Ascaris* or long and filiform resembling *Filaria*. Lips when present, two, paired, simple or trilobate. Oesophagus practically always divided tandem. Vulva more equatorial. Family of interest: Spiruridae.

Spiruridae

Physaloptera caucasica.—Mouth with two equal laterally placed lips, each having three papillae and armed with teeth.

The male has a lancet-shaped posterior extremity and is about ½ inch long (14 mm. by 0.71 mm.). Female is about 1 inch long (27 mm.) with a rounded tail end. Found only once in the alimentary canal of a native in the Caucasus. Leiper has recently reported a species, *P. mordens*, from Uganda, one case. This species is about twice as large as *P. caucasica*.

Gongylonema hominis: A threadlike worm, much like *Filaria*, about 1¼ inches long (32 mm.). The cuticle of the oesophageal zone is provided with shield-like elevations (quite distinct from the bosses of *Loa*). Vulva somewhat preanal. Found in mucous membrane of the mouth and tongue in man. Also found in rodents, cattle, sheep and goats. Cockroaches and croton bugs act as intermediate hosts. Two species reported from Italy are *G. labialis* and *G. subtile*.

FIG. 142.—*Physaloptera mordens*. Mouth parts showing the two lateral lips, each with papillae and teeth. (*After Leiper from Mense.*)

FILARIOIDEA

This superfamily is of the greatest importance to man. It is also one about which much confusion exists as to the adult type; hence anyone finding adult filariae should fix them in hot 5% glycerin alcohol (alcohol 70%), and subsequently mount in glycerin gelatin. Formalin is not to be used, other than for a very brief period (two to six hours) and then followed by the lacto-phenol method. Families:—Filariidae and Dracunculidae.

Filariidae

These worms are most likely to be seen as writhing thread-like worms, especially in the lymphatic glands and connective tissue, and about body cavities. They have a lipped or simple mouth and a filariform oesophagus.

The male has an incurved tail with preanal and postanal papillae which may be even corkscrew-like as in *F. immitis*. The spicules are unequal or there may be but one. The female is usually viviparous; the vulva is at the anterior end and the uterus usually double.

Loa loa (Filaria oculi).—This is a thread-like worm of West Africa; the male is about one inch long, the female about 2–3 inches (50–70

mm.) long and about 0.5 mm. broad. The cuticle is characterized by distinct wart-like structures. These bosses are about 12–15 microns in height. The anterior extremity is like a truncated cone.

The males have four pairs of papillae increasing in size from postanal to preanal, the latter large and pedunculated. There are two short unequal spicules. The

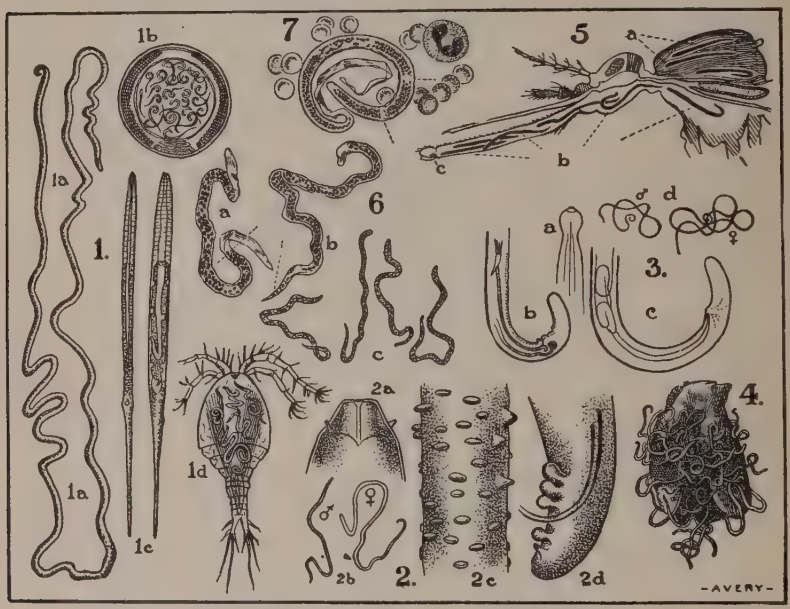


FIG. 143.—(1a) Adult female Guinea worm (*Dracunculus medinensis*) showing anchoring hook at posterior extremity. (1b) Cross section of female *Dracunculus* showing uterus filled with embryos. (1c) Striated embryos of the Guinea worm. (1d) *Cyclops coronatus*, the minute crustacean which serves as the intermediate host of *D. medinensis*. (2a–2d) Anterior and posterior extremities of *Loa loa*. (2c) Section showing tuberculated cuticle. (2b) Male and female *L. loa*, natural size. (3a) Bulbous anterior extremity, *Wuchereria bancrofti*. (3b) Tail of male. (3c) Tail of female. (3d) Male and female, natural size of *W. bancrofti*. 4. Tumor mass of *O. volvulus* laid open. 5. Mosquito showing filarial embryos in thoracic muscles (a) and in labium (b). The labella which are separated from the labium by Dutton's membrane are seen at (c). 6. (a) Embryo of *W. bancrofti* (b) embryo of *L. loa* showing filling of tail end with cells. 7. Microfilaria of *W. bancrofti* in blood. Dotted lines show location in break in cell column and V spot. (*Not drawn to scale.*)

young are born viviparously. It has been suggested that the localized oedemas, known as Calabar swellings, may be due to the mere presence of the adult worm, a toxin, or irritation produced by the larvae. These swellings are of hen's egg size, painless, do not pit on pressure and last about three days. They occur especially on the hands and arms. The embryos almost exactly resemble those of *W. bancrofti*. They have a diurnal periodicity, however, appearing in the blood about 8 A. M.,

increasing to noon and disappearing about 9 P. M. The adult worms have a tendency to wander about in the subcutaneous connective tissue, especially about the region of the orbit or even under the conjunctiva.

Adult worms of *L. loa* have been found and extracted, with an absence of the filarial embryos in the peripheral circulation of the patient. While immature adult worms have been extracted from children the embryos have only exceptionally been found in these children. This would speak for a very long developmental period for the adult worm and as a matter of fact the infection often only shows itself years after the opportunity for infection.

FIG. 144.—*Loa loa* in the subcutaneous tissue, twice normal size. (*From Greene, after Fülleborn.*)

Leiper noted two species of *Chrysops* (mangrove flies) as intermediate hosts, the embryos developing in the salivary glands. Likewise the Connals have noted a development of the larvae in *C. dimidiata* and *C. silacea* similar to that of *W. bancrofti* larvae in the mosquito. The developmental period takes 10

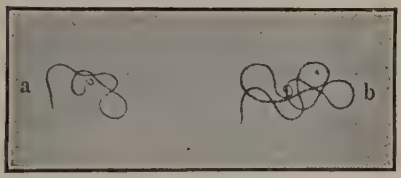


FIG. 145.—Male (a) and female (b) of *Wuchereria bancrofti*. Natural size. (*From Greene, after Manson.*)

days, by which time the larvae, which are now 2 mm. × 25μ with a blunt posterior extremity, are found chiefly at the root of the proboscis. The flies may remain infective for 5 to 6 days.

Wuchereria bancrofti (Filaria bancrofti).—This is the most important of the filarial worms. It is a common infection in south China, India, the West Indies, and in the Pacific Islands, especially Samoa.

In medical books the embryos have been designated *Filaria sanguinis hominis*. This species is the cause of the common manifestations of filariasis, such as elephantiasis, varicose groin glands, chyluria, lymph scrotum, etc.

Filarial diseases are prone to lymphangitis attacks. Thus in lymph scrotum an erysipelatoid condition of the scrotum with high fever and chills may result. This condition is at times mistaken for malaria. Varicose groin glands may be mistaken for hernia. In the Philippines very few symptoms are noted in those affected with filariasis. Occasionally chylocele or chyluria or filarial abscess is reported.

W. bancrofti lives in lymphatics of trunk and extremities. At times the fine white thread-like worms may be seen as writhing coils in lymphatic glands.

The sexes are usually found together often coiled together in cyst-like dilatations of lymphatic vessels or in lymphatic glands. Calcification of these worms is often noted. The females are about 3 inches long and the males less than 2 inches. The tails of both sexes are incurved, but that of the male is more so and has 15 pairs of postanal papillae and two unequal spicules. The head is bougie-shaped with two rows of papillae. The vulva opens 1.2 mm. from the anterior end. There are two uterine tubules. Manson supposed that the sheathed embryos are born vivipa-

FIG. 146.—Section of *Aedes variegatus*, showing filariae in thorax on tenth day of development, travelling forwards into proboscis. (*By permission from Manson's Tropical Diseases.*)

rously and that as a result of injury to the parent worm and resulting extrusion of eggs, the blocking of lymph channels occurs.

Life history.—It is a well known fact that filarial embryos may be present at night in the peripheral blood of persons not showing a single symptom of filariasis and again, in those with marked elephantiasis, varicose groin glands or chyluria there may be an entire and permanent absence of embryos from the blood. When certain mosquitoes bite persons having embryos in the blood they take into their stomachs the sheathed embryos of *W. bancrofti*. Of the proven filarial vectors, *Culex quinquefasciatus*, *Aedes variegatus* and *Aedes albopictus* are the

most important. Complete development of the worm has also been observed in *Anopheles rossi*, *A. ludlowi* and *A. costalis*. Flu states however that in *rossi* and *ludlowi* large numbers of the filarial larvae perish.

The following developmental cycle has been demonstrated for *Culex quinquefasciatus* and *Aedes variegatus*. Bahr has found that if there are too many embryos taken up by the mosquito the insect is apt to die, as the result of too heavy an infection; so that a person harboring many filarial embryos may be less dangerous than one with a smaller number. Upon reaching the stomach of the mosquito the sheath of the embryo becomes fixed in the viscid blood contents and the embryo itself by active motions is able to force itself from its sheath. This escape usually occurs within two hours but may take longer. The free embryo then bores its way through the stomach walls and within twenty-four hours has reached the thoracic muscles of the mosquito. Within forty-eight hours the embryo begins to broaden and the anterior and posterior *V* spots to become more prominent. About the end of the first week there commences the formation of an alimentary canal, by which time the developing larva is about 0.5 mm. long. When the larva is about 0.6 mm. long an ecdysis apparently takes place. Later on these larvae develop 3 or 4 terminal papillae and make their way to the fleshy labium of the mosquito's proboscis. An occasional larva may enter other structures than the labium but in such case they would be unable to effect an entrance to their definitive host, man. These larvae in the proboscis are about 1.5 mm. long and about 20 microns broad.

The mosquitoes have two terminal processes, the labella, separated from the labium by a thin membranous partition called Dutton's membrane. The larvae, having completed their developmental cycle in the mosquito, which takes about three weeks, and moved down the labium, break through this membrane when it is put upon a stretch by the wide separation of the labella at the time of feeding on the part of the mosquito. It was formerly supposed that the larvae entered man through the puncture made by the biting parts of the mosquito, but Bahr has shown by experiments that they effect an entrance through the intact pores of the skin as does the ancylostome larva.

These larvae upon gaining entrance to the circulation of this, its definitive host, proceed to the lymphatic vessels or glands where they attain sexual maturity. The females, having been fertilized by the males, begin giving off sheathed larvae from the uterine opening near the anterior end of the worm. The sheath, which is formed from the egg membrane and develops coextensively with the embryo, appears as a long, narrow sac encasing the fully developed embryo as it exists in man.

From the lymph stream the embryos reach the general circulation. In a case of a man with filarial embryos in his peripheral circulation, who committed suicide one morning, Manson found the embryos, in large part, contained in the vessels of the lungs. There were 675 embryos per slide in blood from the lungs for one from blood from the spleen or liver. It would thus appear that during the day, when the embryos are absent from the peripheral circulation, they retire to the lungs. In the case of the filarial embryo of persons in the Pacific Islands there does not appear to exist any periodicity. Bahr thinks this absence of nocturnal periodicity to be

connected with the habits of its principal intermediary host, *Aedes variegatus*, which feeds by day. *Culex quinquefasciatus* feeds at night.

With the filarial embryos found in patients in the Philippines there is also a lack of nocturnal periodicity. In the opinion of Ashburn and Craig the Philippine filarial worm is a new species, *Filaria philippinensis*.

Walker, however, examined four adult filarial worms in the Philippines and was unable to note any differences from *W. bancrofti*.

Acanthocheilonema perstans (Filaria perstans).—The adults are found in connective tissue and deeper fat, especially about the mesentery and abdominal aorta.

The female is about 3 inches (75 mm.) long; the male, rarely found, is less than 2 inches long. This worm is characterized by an incurved tail, the extremity

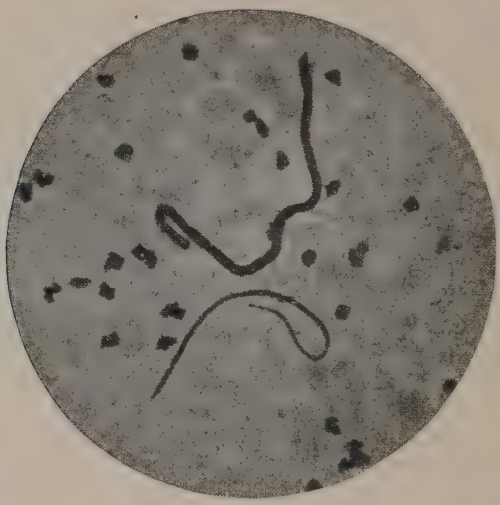


FIG. 147.—Larva of *Loa loa* above; *A. perstans* below. (*From Greene, after Fülleborn.*)

of which has two triangular appendages giving a bifid appearance. The embryo does not possess a sheath and has a blunt tail. The life history is unknown. Both mosquito and fly have been incriminated. The embryos are always present in the peripheral circulation—hence *perstans*. There does not seem to be any symptomatology.

It is of historical interest that *A. perstans* was once considered the cause of sleeping sickness.

Onchocerca volvulus (Filaria volvulus).—This is a rather common parasite of central Africa. A similar parasite has recently been reported from Guatemala. The male is about 1½ inches (35 mm.) and the female about 5 inches long. The females are so interlaced in the fibro-cystic swellings that it is difficult to determine their length. The tumors start from the presence of a worm in a lymphatic. The

tumors are easily enucleated. Adults are striated. They are found in cystic tumors, especially about the axilla and popliteal space. The cystic contents contain abundant sheathless larvae about 300μ long. It was formerly thought that these larvae were absent from the peripheral circulation but more recent investigations have shown a sheathless embryo in the blood of patients with *Onchocerca* nodules which had the characteristics of those found in the contents of the nodules. Life history unknown, although it has been suggested that a species of *Glossina* may be concerned.

Onchocerca caecutiens has been reported from Guatemala. It causes tumefactions and the liberated larvae give rise to an erysipelatoid affection, especially about the face. Its status as a valid species has been questioned.

Recently evidence has suggested a *Simulium* as an intermediate host of *Onchocerca*.

Other Filariae.—*F. ozzardi* (*F. demarquayi*) is a filaria of the West Indies and British Guiana. Its embryo has no sheath and has a sharp tail. Other filarial species which have been reported are *F. magalhaesi, F. powelli, Agamofilaria conjunctivae* and *A. labialis*. The filarial worms of the collective genus *Agamofilaria* are not well determined. They have been reported very rarely from tumor formations or ulcerations. A species called *F. gigas* is now considered to have been only the hair of the leg of a fly.

Embryos.—Embryos have usually been given such names as *F. nocturna, F. diurna*, etc. Of course the embryos and the parent should have the same name. It has been proposed to give these embryos the same designation as the parent, but with the use of the term *Microfilaria* instead of *Filaria* in the same way that *Cysticercus* is used in referring to the larva of *Taenia*.

Larvae of *W. bancrofti* form as a rule graceful curves, those of *Loa loa* angular with an acute bend at the tail. The structure of filarial larvae is best brought out by such stain as dilute Giemsa. The sheath of the larva of *Wuchereria bancrofti* stains easier than that of *Loa loa* and is also somewhat longer. The matrix cells line the body of the larva which is seen to be made up of numerous nuclei. In *W. bancrofti* these nuclei are smaller than in *Loa loa* and do not fill up the tail end as in the latter. The nuclei in *Loa loa* end squarely at the head end whereas in *W. bancrofti* they form a curve. In the body proper of the larvae several breaks may be seen in the column of cells. The first is caused by the nerve ring and is located about ⅙ the length of the body from the head (break in cell column). The V spot is produced by the presence of the excretory pore and the excretory cell. In the posterior end of the larva are seen the large genital cell, three small genital cells and the anal cell. In *Wuchereria bancrofti* there is in addition, a solid structure, located in front of the large genital cell, called the internal body. This is absent in the larva of *Loa loa*.

The points usually noted in the description of filarial embryos are:

1. Presence or absence of periodicity of embryos in peripheral circulation.
2. Presence or absence of a sac sheath around the embryo.
3. Accurate measurements.
4. Shape and description of head and tail ends.
5. Character of movement.
6. Location of V spot and break in cell column in stained specimens.

KEY TO FILARIAL LARVAE IN PERIPHERAL CIRCULATION

A. Sheath present.

1. No periodicity.

F. philippinensis (W. bancrofti?). Tightly fitting sheath; not flattened out beyond extremities. Tail is pointed and abruptly attenuated. Lashing progression movement. 320 × 6.5μ.

2. Periodicity exhibited.

(*a*) Nocturnal periodicity.

W. bancrofti (F. nocturna). Pointed tail; loose sheath; lashing movement. 300 × 7.5μ. V spot 90μ from head; break in cells 50μ from head.

(*b*) Diurnal periodicity.

L. loa (F. diurna). Pointed tail; loose sheath; 245 by 7 microns. V spot 60 to 70 microns from head, break in cells 40 microns from head.

B. Absence of sheath. None of these exhibit a periodicity, being continuously present.

1. Blunt tail—A. perstans. 200 × 4.5μ.
2. Sharply pointed tail:
   (*a*) F. ozzardi. 200 × 5μ.
   (*b*) O. volvulus. 250—300μ × 7.5μ.

NOTE.—A filarial embryo, F. powelli, reported once. It has a sheath, nocturnal periodicity, and is about 130 × 5μ.

Dracunculidae

Differ from the Filariidae in that the vulva appears to be atrophied in the gravid female, the anus is absent and the females are enormously larger than the males.

Dracunculus medinensis (Filaria medinensis).—The Guinea or Medina worm, of which until recently only the female was known, is of great importance in parts of India, Africa and Arabia. The female is a thread-like worm, about 20 to 30 inches long.

The habitat is the subcutaneous and intermuscular connective tissue, especially of the lower extremity. It develops without symptoms. Finally a blister-like area appears on the surface of the leg, particularly about ankle-joint, which soon forms a painful ulcer. From this opening the anterior end of the worm projects to pour forth its striated embryos upon contact with water.

The mouth is terminal and the body uniformly cylindrical. The uterus is a continuous tube filled with sharp-tailed, transversely striated embryos, 650 × 17μ, and constitutes the greater part of the body, the alimentary canal being pressed to one side. The genital organs probably discharge through the oesophagus. The body when being extracted is rather transparent. The tip of the tail is bent, forming a sort of anchoring hook. Leiper fed monkeys on bananas containing infected

Cyclops, and at the autopsy six months later obtained both male and female forms. The two males were quite small, less than 1 inch in length, and were found in the psoas muscle and in the tissue behind the oesophagus.

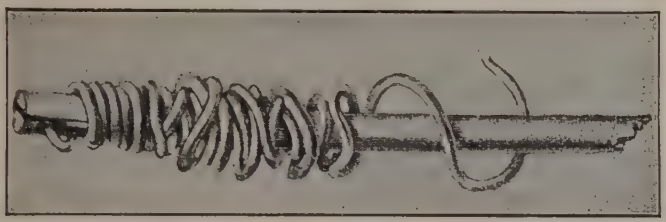


FIG. 148.—Guinea worm. Rolled on a stick for gradual extraction. (*From Greene's Medical Diagnosis.*)

As regards the life history, Fedschenko, in 1870, showed that the embryos when liberated swam around in water and finally entered the bodies of species of the genus *Cyclops*. The female tends to come to the surface in the lower extremities, and

FIG. 149.—Female guinea worm lying under the skin of the forearm. (*By permission from Manson's Tropical Diseases.*)

experiments show that if on the blister-like points of emergence some water be squeezed out from a sponge, the uterus will eject a milky-looking fluid containing myriads of embryos. This would indicate that the worm selects the lower extremity

so that the embryos may gain access to the *Cyclops* when the host is wading through the water. These larvae enter the mouth of the *Cyclops* and after two moults become mature in about five weeks, at which time they are about 1 millimeter long and are found in the thorax of the crustacean.

Leiper showed that a strength of HCl equal to that of gastric juice killed the *Cyclops*, but made the *Dracunculus* embryos very active. From this he judged that infection must probably take place from drinking water containing infected *Cyclops*. The suggestion of Leiper that wells harboring *Cyclops* be treated with steam, introduced by a pipe, seems to be valuable. The disease is known as "Dracontiasis."

GENERAL FILARIAL TABLE

	Adults	Embryos	Remarks
Wuchereria bancrofti (Filaria bancrofti).	Male 40 by 0.1 mm. Female 90 by 0.28 mm. Smooth cuticle. Bulbous anterior extremity. Occupy lymphatic glands and vessels.	Graceful curves; tail rather straight. Sheathed, 300 by 7.5 microns. Distance from head to V spot 90 microns; to break in cells 50 microns. Cells in head end form a curved line. Terminal cells do not fill up tail end. Nocturnal periodicity in peripheral circulation.	Transmitted by mosquitoes. Culex quinquefasciatus and Aedes variegatus. Causes elephantiasis, lymph scrotum, chyluria, etc.
Loa loa.	Male 27 by 0.3 mm. Female 55 by 0.4 mm. Cuticle tuberculated. Anterior extremity like truncated cone. Wanders in subcutaneous tissues.	Angular curves; acute bend at tail. Sheathed, 240 × 7 microns. Distance from head to V spot 65 microns; to break in cells 40 microns. Tail is completely filled up with terminal cells. Diurnal periodicity in peripheral circulation.	Transmitted by species of a biting fly —Chrysops. Causes Calabar swellings. Worms often visit ocular region.
Acanthocheilonema perstans.	Male 40 by 0.07 mm. Female 75 by 0.1 mm. Cuticle smooth. Anterior extremity club shaped. Tip of tail shows two triangular processes. Found about root of mesentery.	Without sheaths, 200 by 5 microns. Posterior two-thirds tapers to bluntending. Cells to end of tail. Distance from head to V spot 49 microns; to break in cells 34 microns. Persists in circulation both day and night.	Transmitting agent not surely known. Mosquitoes and flies suggested. No pathogenicity.
Filaria ozzardi.	Female only is known; 65–80 mm. in length; a pair of fleshy papillae at tail end. Lives in retroperitoneal tissue.	Without sheaths, 200 × 5 mm. Tail sharp pointed; cells not to end of tail.	Transmitting agent. not surely known-Mosquitoes suggested. No pathogenicity.
Onchocerca volvulus.	Male 30 by 0.14 mm. Female usually fragmented. Possibly 75 by 0.36 mm. Cuticle striated. Found coiled up in cyst-like tumors under skin.	Without sheaths. 250 by 7.5 microns. Found in cyst-like spaces of tumors. Found also in peripheral blood and lymph glands.	Method of transmission unknown. Causes small cystic tumors, under skin of thorax especially.
Dracunculus medinensis.	Male from Leiper's monkey 22 mm. Female 80 to 90 cm. long by 1.6 mm. wide. Smooth white body. Anchoring hook at tail end. Female lives in subcutaneous tissue of lower extremity.	Without sheaths. 600 × 20 microns. Tapering outline; gut present. Long slender tail. Cuticle striated. Extruded from break in skin of patient.	Embryos swallowed by Cyclops. Man drinks water containing Cyclops.

Acanthocephala

These are called thorn-headed worms on account of a proboscis which projects anteriorly like a little peg.

There are several rows of hooks surrounding this projection which are directed backward to enable the parasite to attach itself to the intestinal wall. The worm absorbs nourishment through the general body wall, there being no alimentary canal or mouth.

Macracanthorhynchus hirudinaceus (**Gigantorhynchus gigas**).—This parasite is about 6 inches (15 cm.) long for the male and 10 to 12 inches (25 cm.) for the female. It has transverse rings and resembles *Ascaris* but is more white in color. It is said to be not uncommon in southern Russia. The worms are common in hogs. The intermediate host is June bugs. **Moniliformis moniliformis** (**Gigantorhynchus moniliformis**) might be contracted by persons eating death-watch beetles as is sometimes done for the improvement of the complexion. The male is about 2 inches and the female about 4 inches long. A beetle, *Blaps mucronata*, is the usual intermediary. Also found in rats.

Annelida

Leeches belong to the higher worms, class Hirudinea. Members of this class are parasitic and do not possess chaetae, but move about by means of a sucker at the posterior end.

They have a rather oval body marked by numerous rings, and have a well developed muscular system which enables them actively to contract and extend. There is a sucker at the anterior end also; within this is the mouth leading to the pharynx which by the action of its muscular walls serves as a pumping organ. The salivary glands, situated inside the mouth cavity, secrete the fluid which prevents coagulation of the blood. The mouth may or may not be provided with cutting jaws. In *Hirudo* there are three semicircular jaws the arched surfaces of which are beset with from 50 to 100 sharp teeth. The mark of a leech bite is triangular. When a leech has gorged itself, it becomes detached from the skin of its victim, but the effect of the salivary secretion in retarding coagulation is of some duration so that the wound continues to bleed. With some of the leeches the wounds frequently become infected and ulcers which may prove serious, often result. This is particularly true with the *Haemadipsa zeylanica*. This species is a land leech but requires abundant moisture. Most leeches live in water.

As a rule leeches are hermaphroditic and reproduce by depositing so-called cocoons, which are rounded bodies surrounded by a shell and containing eggs in an albuminous matrix.

Hirudo medicinalis.—This is the leech used medically for the abstraction of blood. They have a secretion which prevents coagulation of the blood so that when they are removed the wound still continues to bleed. These leeches are about 4 inches long and of a grayish-green color with dingy red longitudinal stripes on the dorsal surface and with a dark-green ventral surface.

Limnatis nilotica.—This species has been found in many parts of northern Africa. Gaining access to the mouth through drinking water, it wanders to the pharynx, nares, and even trachea. Manson refers to a case of obstinate epistaxis and headache caused by a leech in the nostril.

This leech is about 4 inches long (8 to 10 cm.) and about ½ inch (1.2 cm.) broad. The dorsal surface is greenish-brown with narrow orange-brown borders. The young leeches are only about ⅛ inch (3 mm.) long and taken in with the drinking water may attach themselves to the surface of some mucous membrane and after some weeks reach adult size.

Haemadipsa zeylanica.—These are land leeches found in India, Philippines, Australia, and South America. They are only about 1 inch (25 mm.) long and are slender. They leave the damp earth to climb shrubs and from there to drop on animals or man passing through the forest. Their bites are painless, but may be followed by ulcers. They may get into the nostrils.

They will even penetrate thick clothing in order to reach the skin.

CHAPTER XX

THE ARACHNOIDS

CLASSIFICATION OF THE ARACHNOIDEA

Order	Family	Genus	Species
Acarina	Trombidiidae	Trombicula	T. akamushi
		Trombidium	T. holosericeum
	Parasitidae	Dermanyssus	D. gallinae
	Tyroglyphidae	Tyroglyphus	T. farinae
			T. longior
		Glyciphagus	G. domesticus
	Sarcoptidae	Sarcoptes	S. scabiei
	Demodicidae	Demodex	D. folliculorum
	Pediculoididae	Pediculoides	P. ventricosus
	Argasidae	Argas	A. persicus
			A. miniatus
		Ornithodoros	O. savignyi
			O. moubata
	Ixodidae	Ixodes	I. ricinus
		Hyalomma	H. aegyptium
		Rhipicephalus	R. bursa
		Dermacentor	D. reticulatus
			D. andersoni
		Boophilus	B. annulatus
		Amblyomma	A. hebraeum
		Haemaphysalis	H. leachi
Linguatulida	Linguatulidae	Linguatula	L. serrata
		Armillifer	A. armillatus

The class Arachnoidea and the class Insecta belong to the phylum Arthropoda. This phylum contains a greater number of species than does any other phylum; in fact it may be said to exceed in this respect all other phyla combined.

Other arthropods are the Myriapoda, or thousand-legged worms, and the Crustacea, to which belong the lobsters, crabs and water-fleas—important zoologically but of very slight importance medically. For Venomous Arthropods, see page 572.

The different classes of Arthropoda resemble the segmented worms but have as the point of distinction the possession of jointed appendages

which proceed from the somites in pairs. Some of the pairs of limbs are for locomotion; at times, certain ones may be specialized for food taking.

The somites or divisions of the body have a chitinous exoskeleton. Respiration takes place through the medium of gills in the Crustacea and by tracheal tubes in the Myriapoda, Arachnoidea, and Insecta.

The Arachnoidea have no antennae while the Myriapoda and Insecta have a single pair of antennae, the former having numerous pairs of legs or jointed appendages while the latter have only three pairs of legs. The Arthropoda have segmented bodies, but they differ from the worms in having jointed appendages for the purpose of taking in food and moving from place to place. They also have an exoskeleton which is more or less unyielding from the deposit of chitin in the cuticle. This cuticle is not true skin but only a secretion of the epidermis.

Within this external skeleton we have a dorsal digestive system and a ventral nervous system.

Great importance of arthropods in medicine.—Members of this phylum are important not only because of certain immediate and direct effects of their activities, such as the action of poisons introduced by scorpions, spiders and ticks, or the painful and peace-disturbing attacks of various biting arachnoids and insects, but in vastly greater degree in that among them are our most important transmitting agents of disease. The following is a list of the diseases transmitted by them.

Transmitted by arachnoids.—Rocky Mountain spotted fever, tsutsugamushi, tick-bite fever and the relapsing fevers of East and West Africa and of Panama.

Transmitted by insects.—Typhus fever, European and Indian relapsing fevers, trench fever, American and African trypanosomiasis, plague, tularaemia, filariasis and loaiasis, malaria, yellow fever, pappataci fever, dengue, oriental sore and possibly other forms of leishmaniasis, together with certain helminthic infections (*Dipylidium* and *Acanthocephala*).

Transmitted through crustaceans.—Infections with *Dracunculus medinensis* and *Diphyllobothrium latum* through the medium of *Cyclops*, and paragonimiasis through certain species of crabs.

There are many arthropods which may accidentally bring about direct transference of disease, as with tabanid or stable flies which, following contamination of their biting parts with anthrax bacillus blood, might directly transfer the virus, when shortly afterward feeding on a man or animal. Many non-biting flies, in particular the house fly, and possibly cockroaches or other arthropods having access to our food or faeces, are important agents in the spread of typhoid, cholera, bacillary

dysentery, and amoebic dysentery. The itch mite and the sand flea, as well as certain fly larvae, by burrowing into the skin or sinus mucous membranes, are well recognized causes of disease. Fly larvae are also the causes of various intestinal myiases. Bacterial infections of mosquito or other insect wounds, by scratching, are frequently reported.

On pages 385 and 386 will be found a list of arthropodan diseases.

THE ARACHNOIDEA

The Arachnoidea differ from the Insecta in having the head and thorax fused together. They also have four pairs of ambulatory append-

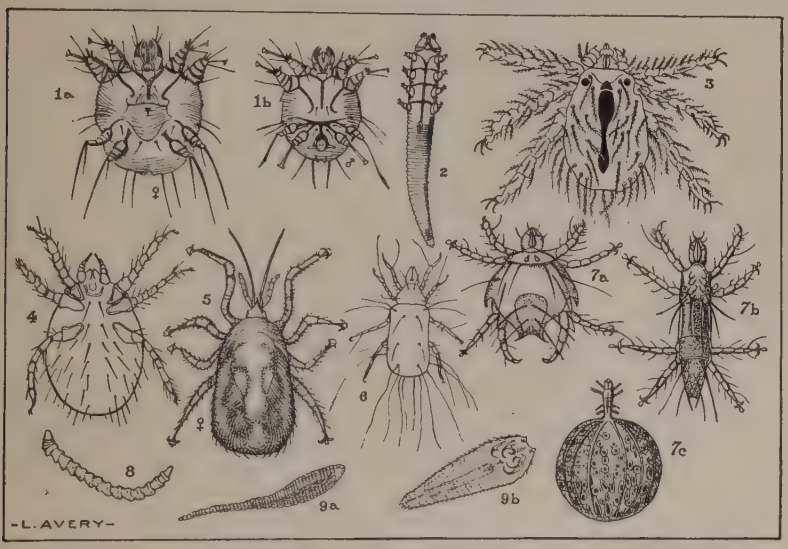


FIG. 150.—Arachnoidea exclusive of ticks. (1a) *Sarcoptes scabiei*, female; (1b) *S. scabiei*, male; (2) *Demodex folliculorum;* (3) *Trombicula akamushi*, hexapod larva (Kedani mite); (4) *Trombidium holosericeum* larva (*Leptus*); (5) *Dermanyssus gallinae;* (6) *Tyroglyphus longior;* (7a) *Pediculoides ventricosus*, male; (7b) *P. ventricosus*, young female; (7c) *P. ventricosus*, impregnated female; (8) *Armillifer armillatus;* (9a) *Linguatula serrata*, female; (9b) *L. serrata*, larva. Note: Figure not drawn to scale.

ages, while the insects have three pairs. The Arachnoidea never have compound eyes—eyes when present being simple. Of the two orders of Arachnoidea of interest medically the Acarina is far more important than the Linguatulida.

ACARINA

Of the acarines we are chiefly interested in the mites and the ticks. The acarines do not show any separation of the abdomen from the

cephalo-thorax. A hexapod larva develops from the egg; this is succeeded by an octopod nymph which differs from the adult in not having sexual organs.

In addition to the four pairs of legs in the fully developed acarine there are two other paired appendages, the chelicerae, in front of the mouth, and the pedipalps on either side of the mouth.

Trombidiidae

These generally have a soft, more or less hairy integument and are often brightly colored. The two eyes are often pedunculated and the chelicerae are lancet-shaped and the palps project beyond the rostrum as claw-like appendages. A tip-like appendage on the apical segment of the palps is characteristic. A very common and annoying member of this family is the hexapod larva of the *Trombidium holosericeum*. It is usually designated *Leptus autumnalis*. Popularly it is termed "harvest mite," "red bug" or "jigger." They are found in the fields in the autumn and attack both man and animals. The condition (itching and redness) produced is at times called autumnal erythema. There is a *Trombidium* in Mexico which has a predilection for the skin of the eyelids, prepuce, and navel.

Trombicula akamushi (Microtrombidum akamushi).—The genus *Trombicula* differs from *Trombidium* in having 2 claws on distal segment of palp. It is called the Kedani mite. It is an orange-red larval mite about 400 by 200 microns. It is believed by the Japanese authorities to bring about infection with Japanese river fever or tsutsugamushi, as the result of transmitting either a bacterium or protozoon by its bite. These mites are very hairy and live inside the ears of the field mice of the area of Japan in which the disease occurs. The adult forms live in the soil of the endemic area. They are about 1 mm. long by 0.5 mm. wide. The disease somewhat resembles typhus, although an eschar marks the site of the bite and lymphatic involvement is present.

PARASITIDAE

Of the Parasitidae, which generally have a hard leathery body and styliform piercing chelicerae, delicate five jointed pedipalps and styliform hypostome, only the *Dermanyssus gallinae* is of interest. This mite infests chicken-houses and sucks the blood of the inmates. They will also attack man. Poultrymen may be troubled with a sort of eczema on the backs of the hands and forearms, similar to scabies, resulting from bites by these mites. They measure $350 \times 650\mu$. They have no eyes.

Tyroglyphidae

Mites of this family live on cheese, flour, dried fruits, etc. They are small, without eyes, and have a smooth skin and a cone-like appearance of the mouth parts which are largely formed by the chelate chelicerae. They are chiefly of importance because of their being occasionally found in urine, faeces, etc., and being striking objects, the question of pathogenicity arises. The *Tyroglyphus longior* has been associated with intestinal trouble (probably a coincidence, patient having eaten cheese containing these mites).

Glyciphagi are found in sugar and are the cause of what is known as "grocer's itch." *Rhizoglyphus parasiticus* is reported to be the cause of an itch-like affection of the feet of coolies on tea plantations. To distinguish: The dorsum and legs of *Glyciphagus* are covered with plumose hairs; *Tyroglyphus* has both claws and suckers on tarsi, while *Rhizoglyphus* has only claws.

Sarcoptidae

These are small eyeless mites with a transversely striated cuticle. They live on the epidermis of man and various animals. The rostrum is chiefly made up of chelate chelicerae with quite short three-jointed, rather adherent palpi. It is the female that makes the tunnels in the skin between the fingers, on penis, flexor surface of forearm, etc. The male dies off after copulation. The female passes through four stages: (1) Larva; (2) nymph; resembles adult, but has no sexual organs; (3) the pubescent female; (4) the egg-bearing female. A pair of itch mites may produce 1,500,000 descendants in three months. Transference of eggs, larvae or pubescent females does not seem to transmit scabies; it is the egg-laden female only. The human itch mite, *Sarcoptes scabiei*, is an oval mite; the male is 250 × 150μ, the female about 400 × 300μ. Besides the difference in size, the male may be distinguished from the female by the fact that the third and fourth pairs of legs in the female have bristles, but in the male, the fourth pair has suckers. The tunnels made by the female have the egg-bearing female at the blind end; scattered all along are faeces, eggs and larvae, the eggs being next the mother and the more mature young at the entrance to the gallery. A diagnosis can be made from the finding of either eggs or larvae. The eggs, 140μ long, hatch out in four to five days. A female becomes mature in about two weeks.

In treating itch with sulphur preparations the adult females and immature itch mites are killed; the eggs, however, are not affected. Hence a second treatment about ten days after the first is necessary to kill the young mites, which have developed subsequently to the first treatment. Different animals have different species of itch mites.

Demodicidae (Hair Follicle Mites)

Demodex folliculorum.—This is a vermiform acarine about 400μ long, living chiefly in the sebaceous glands of nose and forehead. The eggs are about 75 × 35μ. A six-legged larva hatches from the egg and develops into an eight-legged adult after four moultings. Some of the cases of "blackheads" are due to this face mite. Another species of *Demodex* causes a severe mange in dogs.

PEDICULOIDIDAE

This acarine family shows a complete dimorphism. The last two pairs of legs are widely separated from the front legs. The *Pediculoides ventricosus* is oval and about 125 × 75μ for the male which has claws at the extremities of the anterior and posterior pairs of legs; the two other pairs have hooklets and a sucking disc. The female is about twice as long but of the same breadth as the male, and has claws only on the anterior legs. The chelicerae are lancet shaped and retractile. The

large pedipalps are fused together anteriorly. The gravid female is like a ball and is about 1000μ in diameter.

They live on wheat and may be found in wheat straw, handling of which may be followed by a severe skin eruption with an irregular fever. These mites are viviparous.

Ixodoidea

This family of the Arachnoidea is one of great medical interest and of growing importance. It is divided into the families Argasidae and Ixodidae.

While proven the intermediary hosts only in the case of the organisms of African tick fever, relapsing fever of Panama and spotted fever of the Rocky Mountains, it has been considered possible that blackwater fever also belongs in this category on the assumptions that it is caused by *Babesia* (*Piroplasma*) and that piroplasmata in man are, as is almost invariably the case with animals, transmitted by ticks.

Very important diseases due to these small pear-shaped organisms within red cells are known for various animals, the best known being that of cattle in Texas and termed Texas fever. Other piroplasmata diseases are Rhodesian fever (cattle), heart water (sheep), and malignant jaundice of dogs. In these diseases there are pathological features which resemble blackwater fever of man.

It is of interest to note that it was with the transmission of Texas fever through an intermediate host (the tick) that Smith and Kilborne (1889–1893) established the zoological principle of transmission of disease through arthropod intermediary hosts. This led up to the work on malaria, yellow fever, etc.

Ticks differ from insects in having four pairs of legs, only two pairs of mouth parts, and no antennae. They differ from other acarines in having a median probe-shaped puncturing organ, the hypostome, which is beset with numerous teeth projecting backward, and in possessing stigmal plates. The head, or capitulum, or rostrum, is the part which projects anteriorly from the body. This carries the piercing parts which are the single hypostome or dart and a pair of piercing chitinous structures, the chelicerae, which lie above the hypostome. As a sheath for these delicate biting parts we have a segmented pair of palpi or pedipalps. The mouth is a slit between the chelicerae and hypostome.

Two depressed pitted areas on the dorsal surface of the capitulum in the adult female are known as porose areas. Very important structures are the stigmal plates. These are striking mosaic-like areas which are located just posterior to each hind leg in the Ixodidae and between the third and fourth legs in the Argasidae. Great confusion has existed in the generic and the specific classification of ticks, but the group is now rapidly being brought into more satisfactory order. The microscopic structure of the stigmal plates has been shown by Stiles to be of extreme value in differentiating the various species, especially of *Dermacentor*. The stigmal orifice, the opening of the tracheal system, is in the center. The Ixodidae

have a scutum or shield-like chitinous structure on the dorsal surface. It covers almost the entire back of the tick in the male but only a small portion anteriorly in the female. The genital opening is toward the anterior part of the ventral surface. The anus, with anterior or posterior anal grooves, is near the posterior third of the venter. The legs have six segments, the coxa being flattened out on the surface of the body and the terminal tarsus ending with a pair of hooks and at times with a pulvillus. The nymph has stigmal plates but has no genital opening while the larva has neither genital apertures nor stigmal orifice.

Life History of Ticks.—This varies greatly according to the subfamily, genus, and species. The larva of *Ornithodoros moubata* does not leave the egg, but moults inside, and finally emerges as an eight-legged nymph. It lives in the dust in the cracks of the native huts and comes out at night to feed on the sleeping natives.

With some of the Ixodidae the females lay from 5000 to 20,000 eggs during several days or weeks; but with the Argasidae, the possibilities of destruction being less, there is not the same necessity for producing thousands of eggs to ensure continua-

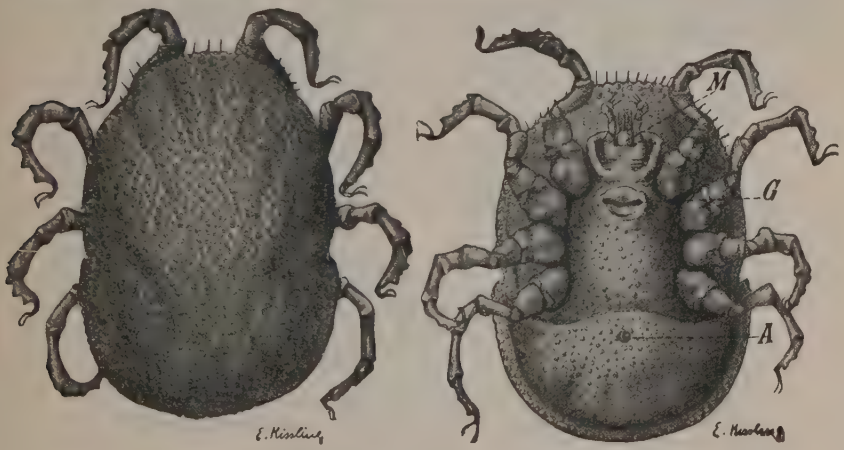


FIG. 151.—*Ornithodoros moubata.* (*Murray from Doflein.*)

tion of the species. The female *Ornithodoros savignyi* for example lays only about 140 eggs. The eggs of ticks are preferably deposited near grass. The egg stage lasts from one to six months, when the six-legged larva ("seed tick") emerges. It crawls up a blade of grass and gets on a passing animal. After feeding, or at times without taking nourishment, the larva drops to the ground, and changes to the nymph which has four pairs of legs. The nymph crawls up a blade of grass and gets on a passing animal (the second host). Having fed, it falls to the ground where it remains eight or ten weeks. It moults and develops into an adult tick. The adults gain access to a third animal host—the males fecundate the females, after which the

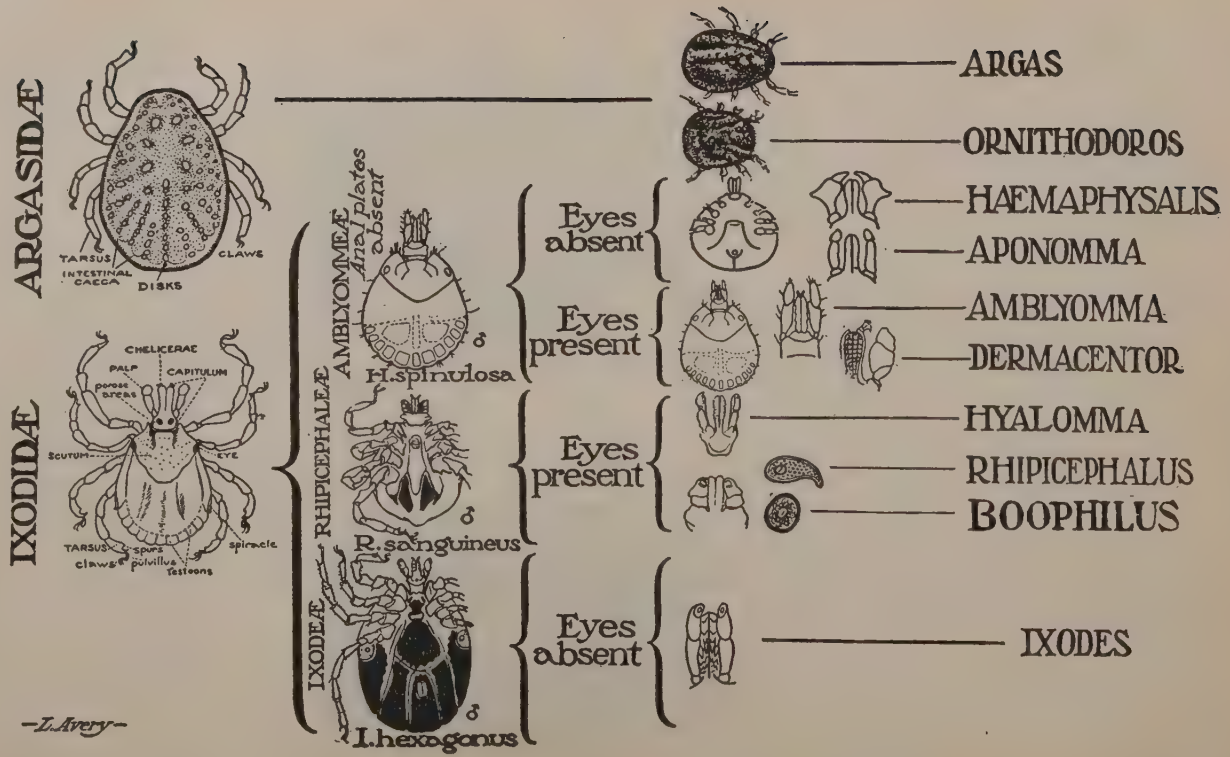


FIG. 152.—Diagrammatic key to ticks, modified from Lahille. Includes Ixodeae. (Males clothed on all their ventral surface with anal plates in uneven numbers. Rostrum elongate. No eyes.) Rhipicephaleae. (Males having anal plates in pairs. Rostrum may be long or short Eyes present.) Amblyommeae. (Males without anal plates.)

female gorges herself with blood, subsequently dropping off the animal and laying eggs. With some ticks fewer hosts suffice.

Cleland has noted reports of serious symptoms, chiefly cardiac and visual, from the bite of ticks in Australia (*Ixodes holocyclus*). This is exceptional, however, as the symptoms following the bites of such ticks are usually only those of skin irritation.

Classification of Ixodoidea

FAMILY ARGASIDAE.—Head concealed by body when viewed dorsally. No scutum. Stigmal plates between third and fourth legs. Adults have no suckers (pulvillus) beneath claws. Slight sexual dimorphism. Anus near middle of venter. Skin rough.

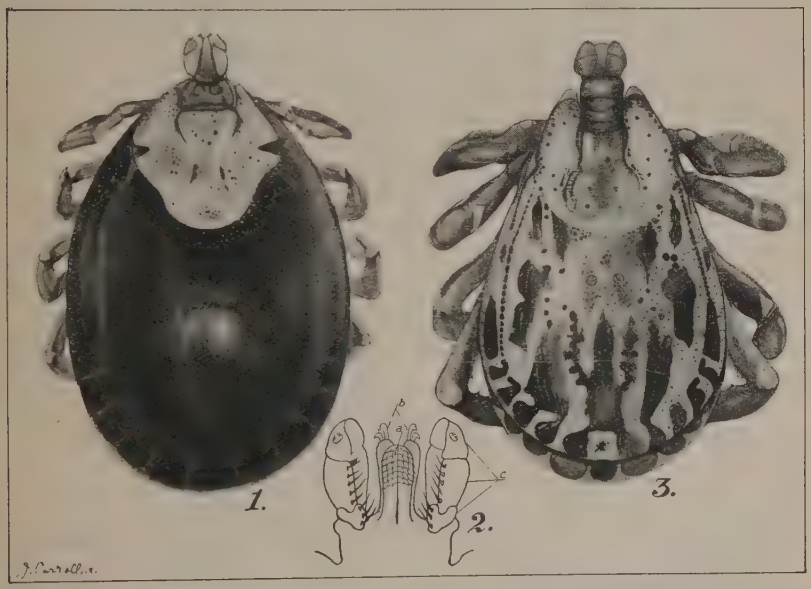


FIG. 153.—1. Female *D. andersoni*. 2. Head showing (*a*) hypostome, (*b*) chelicerae, (*c*) palps. 3. Male.

Genus Argas.—Body narrow in front. Margins thin and acute. No eyes. The *A. persicus* (Miana bug) of Persia has been supposed to be concerned in the transmission of a form of relapsing fever. Rostrum some distance from anterior margin. It is also called the fowl tick and transmits fowl spirillosis.

Genus Ornithodoros.—Margins of body rounded. Skin has many irregular tubercles. Rostrum even with anterior margin so that ends of palpi slightly project. *O. talaje* transmits the relapsing fever of Panama. *O. moubata* is the intermediate host of *Borrelia duttoni* (South African tick fever).

O. moubata is very common in Africa living in cracks in mud floors whence it issues to bite severely the sleeping natives. The larva makes its first moult inside the egg so that it emerges as an octopod nymph. Christy thinks it may transmit *Acanthocheilonema perstans*.

O. savignyi has two pairs of eyes, one pair near base of mouth parts and the other between the second and third coxae.

Tick fever.—With tick fever the epidemiology rests upon the life history of the tick *O. moubata*. This tick infests the rest houses along the route of travel, hiding in the crevices of floors and walls during the day and coming out at night to bite the sleeping inmates. The feeding occupies a long time, more than an hour. Both sexes bite man.

The female lays about 100 eggs, from which a nymph emerges in about twenty days. The larval stage takes place in the egg. Shortly after emerging the nymphs suck blood. An important fact is that the female transmits the spirochaete to its ova, so that the nymphs may transmit the disease.

Natives seem to suffer severely from tick fever in childhood but in adult life possess a sufficient degree of immunity so that the disease shows itself in a very mild form in those harboring spirochaetes. Ticks can be infected by these carriers. In some of the rest houses 50% of the ticks may be infected. While the tick does not tend to leave its habitation it may be transported in the bundles of native porters.

The transmitting agent of the North African relapsing fever and probably of the Indian type is the louse.

Family Ixodidae.—Mouth parts project in front of body when viewed dorsally. Scutum present. Stigmal plates posterior to fourth pair of legs. Adults have suckers beneath claws. Skin finely striated. Anus behind middle of venter. Sexual dimorphism marked. Male has well developed scutum; female has porose areas.

Section Ixodeae.—Transverse recurved preanal groove in female. Male has ventral surface covered with chitinous plates. No eyes. Genus *Ixodes*.

Ixodes has long rostrum with slender palpi—palpi narrow at base, leaving gap between them and hypostome.

Section Rhipicephaleae.—No preanal, but postanal groove in female. Ventral surface of male without adanal plates in *Dermacentor*, *Haemaphysalis*, *Aponomma* and *Amblyomma*, but with one or two pairs in *Hyalomma*, *Rhicephalus* and *Boophilus*.

In the genera *Hyalomma*, *Aponomma* and *Amblyomma* the palpi are long and slender and of about uniform width of segments.

In *Hyalomma* the segments of palpi are of about equal length. In *Aponomma* and *Amblyomma* the second palpal segment is much longer than the others. *Amblyomma* differs from *Aponomma* in being very ornate and in having eyes.

In the genera *Haemaphysalis*, *Dermacentor*, *Rhipicephalus*, and *Boophilus* the palpi are short.

Haemaphysalis has very broad rostrum, triangular palpi, and no eyes. *Dermacentor* has a square rostrum with short thick palpi, the second and third joints being

as broad as long. *Dermacentor andersoni* transmits spotted fever of the Rocky Mountains—not *D. reticulatus*. Rocky Mountain fever is discussed in Chapter XXXVIII. In British Columbia the bite of this tick is said to cause a paralysis. The most important characteristic of the genus *Dermacentor* is the large size of the coxae of the fourth pair of legs.

Dermacentor andersoni.—The male is about 2.5 × 4 mm. The female has a conspicuous whitish scutum 1.6 mm. long by 1.9 mm. broad. The young female is about 6 × 2.5.—the replete female about 15 × 9 mm. Both male and female ticks transmit the disease as well as larval and nymphal forms. The adult ticks live on horses and cattle while the young forms infest the rodents of the district. The female deposits about 6000 eggs and the life cycle takes about two years.

Many authors name the tick transmitting Rocky Mountain fever the *D. venustus*. Stiles considers this term to apply to a Texas tick not connected with the disease, and believes *D. andersoni* to be the proper name of the vector. *D. andersoni* also transmits tularaemia.

Rhipicephalus has palpi without transverse ridges and comma-shaped stigmal plates. The stigmal plates of *Boophilus* are nearly circular and the palpi have acute transverse ridges externally. *Boophilus annulatus* transmits Texas fever of cattle. This tick is also called *Margaropus bovis* or *M. annulatus*. Some authors term it *Rhipicephalus annulatus*. Larvae developing from eggs of female ticks which have fed on cattle infected with Texas fever transmit the disease which is due to a protozoon *Babesia bigemina*.

Linguatulida (Tongue Worms)

These are vermiform arachnoids more or less distinctly annulated. They have retractile hooks at either side of the elliptical mouth.

If the hooks are to be considered not as degenerated legs but antennae and palpi, then there is no vestige of legs in the adult. The sexes are separate.

Linguatula serrata (L. rhinaria).—This has been observed in man both in larva and adult stages. The nymph is also known under the name *Pentastoma denticulatum*.

The male is white and about ¾ inch long while the female is about 4 inches long, tadpole shape, yellowish in color, and has about 90 segments. It lives in the nasal cavity and frontal sinus of carnivorous animals, such as dogs, rarely in horses and sheep, and very rarely in man. Some herbivorous animal serves as the intermediate host.

The female lays embryo-containing eggs which, discharged with the nasal mucus, are swallowed by various animals (rabbit, sheep). A larva develops which bores its way through the gut and encysts in the liver or mesenteric glands. Here the larva undergoes a series of moultings. When the viscera of the intermediate host are eaten by some carnivorous animal the encysted larvae are liberated in the stomach and find their way to the nasal passages of the new host, where adult forms develop.

The larval form (⅛ inch) is far more common in man than the adult and is most often found in the liver. Symptoms are referred to liver in infections with both larval and adult stages, and epistaxis and nasal symptoms for the adult stage only.

Armillifer armillatus (Porocephalus armillatus).—The adult form lives in the trachea or lungs of African snakes. The male is 1 to 1.5 inches long and shows 16–17 rings. The female is 3 to 4 inches long and has 18–22 rings. The eggs are probably ingested by an intermediate host (rats, cats, monkeys) through drinking-water or food, contaminated with discharges from the snake. These eggs develop into curled-up ringed larvae, about ½ inch long with the same number of rings as the adult, and become encysted especially in the liver or lungs. The larvae escape and are swallowed by the snakes, their definitive hosts (probably snake devours the intermediate host), and reach the lungs where the adult forms develop.

The larval stage has been found in man and may then give rise to bronchitis, hepatitis or peritonitis. Cases are usually only discovered postmortem.

CHAPTER XXI

THE INSECTS

CLASSIFICATION OF THE CLASS INSECTA

ORDER	FAMILY	SUB-FAMILY	TRIBE	GENUS	SPECIES
Anoplura	Pediculidae			Pediculus	P. humanus
					P. vestimenti
				Phthirus	P. pubis
Hemiptera	Cimicidæ			Cimex	C. lectularius
					C. rotundatus
	Reduviidae			Triatoma	T. megista
					T. sanguisuga
Siphonaptera	Pulicidae			Pulex	P. irritans
				Xenopsylla	X. cheopis
	Archaropsyllidae			Ctenocephalus	C. canis
					C. felis
	Histrichopsyllidae			Leptopsylla	L. musculi
	Dolichopsyllidae			Ceratophyllus	C. fasciatus
				Hoplopsyllus	H. anomalus
	Tungidae			Tunga	T. penetrans
Diptera	Tabanidae			Tabanus	T. autumnalis
				Haematopota	H. pluvialis
				Pangonius	P. beckeri
				Chrysops	C. discalis
				Glossina	G. palpalis
					G. morsitans
	Muscidae			Stomoxys	S. calcitrans
				Musca	M. domestica
				Auchmeromyia	A. luteola
				Calliphora	C. vomitoria
				Lucilia	L. caesar
				Chrysomyia	C. macellaria
				Cordylobia	C. anthropophaga
	Sarcophagidae			Sarcophaga	S. carnaria
	Oestridae			Dermatobia	D. hominis
				Hypoderma	H. diana
	Simuliidae			Simulium	S. reptans
	Psychodidae			Phlebotomus	P. papatasii
	Chironomidae			Ceratopogon	C. pulicaris
	Culicidae	Culicinae	Sabethini	Wyeomyia	W. smithii
			Culicini	Aedes	A. aegypti
				Culex	C. quinquefasciatus
			Anophelini	Anopheles	A. maculipennis
		Corethrinae		Corethra	C. cinctipes

Insecta

The class Insecta has one pair of antennae, three pairs of mouth parts (the fused labium being considered as one pair), and three pairs of

legs. They have three divisions of the body—head, thorax, and abdomen.

The head carries the antennae and mouth parts; the thorax, which is divided into the prothorax, mesothorax and metathorax, carries upon the ventral surface of each thoracic segment a pair of legs and on the dorsal surfaces of the two posterior segments a pair of wings. The abdomen does not support appendages. The air is supplied by means of tracheae —branching breathing tubes which have external openings or stigmata. The tracheae are stiffened by spiral chitinous bands. The Malpighian tubules are excretory organs of the alimentary system and excrete nitrogenous waste material. Insects have two pairs of wings, the second pair of which is frequently rudimentary and shows simply as knob-like projections. These are termed halteres or balancers. In some insects both pairs of wings are rudimentary, as in Siphonaptera.

When insects show metamorphosis we have voracious worm-like larvae coming out of eggs; these larvae are succeeded by a quiescent nonfeeding encased pupa which finally develops into an imago or fully developed insect. An insect which does not present this developmental cycle shows incomplete metamorphosis. Of the class Insecta only the Anoplura, Hemiptera, Siphonaptera, and Diptera are of special importance.

Anoplura (Siphunculata)

These are small dorso-ventrally flattened wingless insects not showing metamorphosis.

Pediculidae

In this family there are no wings and there is no metamorphosis. They have simple eyes and 5 joints to the antennae. The legs are well developed and terminate in powerful claws. The young resemble the adults. The acorn-shaped eggs (nits) are deposited on hairs or clothing of the host.

Pediculus humanus (**Pediculus capitis**).—The eggs, usually 60 in number, are deposited on the hairs of the head, the favorite region being back of the ears. They hatch out in about six days. The lice larvae on emergence closely resemble the adult and begin to feed shortly after hatching. They moult about every three days and become adults within ten days.

The adults vary in color according to the color of the hair of the host. The thorax is as broad as the abdomen. The male louse is smaller, is rounded off posteriorly and shows a dorsal aperture for a pointed penis; while the female is recognized by its larger size, 2 mm. in length, and by a deep notch at the apex of the last abdominal segment.

There seems to be a marked preference exhibited by lice for their own peculiar racial host. It has been suggested that this might account for certain peculiarities in infection where different races were living together and under similar conditions

as to food and environment, and yet only one race contracts the disease. The head louse has been found to harbor leprosy bacilli when living on a leper.

Pediculus vestimenti (**P. corporis**).—This louse lives about the neck and trunk underclothing, being rarely found on the skin. The louse feeds about twice a day, deprivation of food killing the adult in nine days and the newly hatched louse in two days.

The female, under favorable temperature conditions (65°F.), begins to oviposit three or four days after reaching maturity, and thereafter, during her average life of four or five weeks, lays four or five eggs daily.

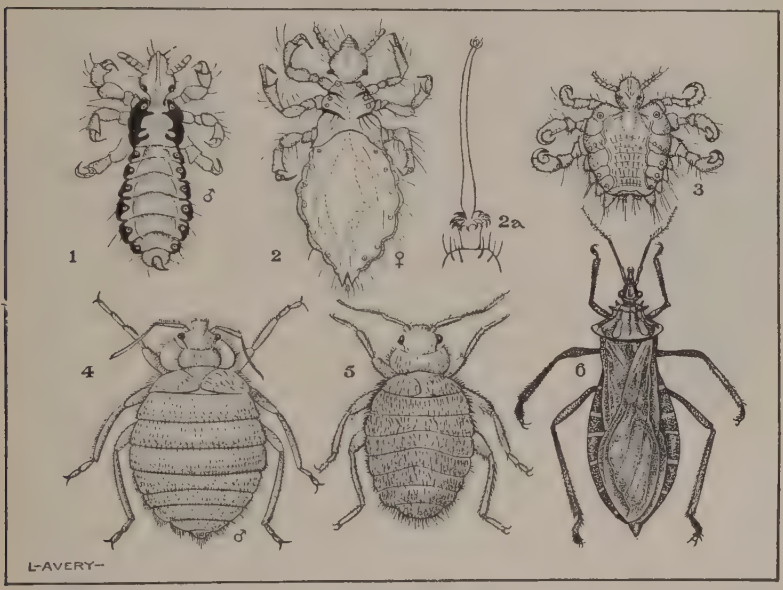


FIG. 154.—Anoplura and Hemiptera. 1. *Pediculus humanus.* 2. *Pediculus vestimenti.* 2a. Protruded rostrum of *Pediculus.* 3. *Phthirus pubis.* 4. *Cimex lectularius.* 5. *C. rotundatus.* 6. *Triatoma megista.*

The eggs, when on clothing next the body, hatch out in 7–10 days, and become mature in about two weeks. There is no grub stage as with the fleas.

As the eggs are usually deposited in inaccessible portions of the clothing, as in the seams, and since they remain viable there for more than a month, infested clothing should be steam-treated before being worn.

Anatomy.—The body louse is somewhat larger than the head louse and there is less marked festooning of the sides of the abdomen so that the segments are less well marked. The head is separated from the thorax by a narrow neck, there being but slight differentiation between the thorax and abdomen. The head is rather olive-shaped and more pointed anteriorly where is situated the mouth surrounded by a hook-bearing ring (the haustellum). The five-jointed antennae are attached

to the side of the head. The three stylets are inside the mouth and are long and sharp. They can be protruded through the mouth orifice and when apposed make a tube through which the secretions from the salivary glands empty. These needle-like stylets make the wound which causes the blood to flow. The blood is sucked up by the pharynx which lies above the stylets and has muscular walls making it a pumping organ. From this a narrow oesophagus leads into the long prominent stomach which terminates in a narrow S-shaped hind-gut. The tracheal system (respiratory) opens along the sides of thorax and abdomen in round openings or spiracles. These can be closed by oils, thus asphyxiating the louse. The female has a pouch-like opening beneath the hind-gut which leads to the oviduct which connects with two ovaries. The eggs receive the cement material in the oviduct.

Fig. 155.—Female *Pediculus vestimenti*. (*Schamberg, after Kuechenmeister.*)

The body louse has been shown to transmit typhus fever and Nicolle has demonstrated it as a carrier of relapsing fever. The spirochaetes are introduced, not by the act of biting but subsequently as a result of scratching whereby infective material from a louse is rubbed into the wound. Trench fever also is transmitted by the body louse and is inoculated in the same manner; but it is to be noted that in case of relapsing fever the virus is derived from the crushed louse (body fluid) whereas in the case of trench fever it is more commonly found in the faeces. These viruses apparently undergo some developmental cycle in the louse, since the louse is incapable of transmitting these diseases until several days have elapsed following the infecting feeding: Typhus fever, 10 days; relapsing fever, 4 days; trench fever, faeces infective after 7 days. The dog louse as well as the dog flea serves as an intermediary host for *Dipylidium*.

As the body and head louse differ more in habit or location than in structure and will interbreed readily, some observers regard them as varieties of the same species, i.e., *Pediculus humanus*, varieties *humanus* and *corporis*.

Phthirus pubis.—This louse is popularly known as the crab louse. The female is little more than $\frac{1}{25}$ inch in length, and the male a trifle less. They are almost square. The second and third pair of legs are supplied with formidable hooks. They have a preference for the white race and live about the pubic region. The female lays about a dozen eggs, which hatch out in about a week.

Hemiptera (Rhynchota)

The Hemiptera or bugs are insects possessing mouth-parts modified for sucking in which the lower lip or labium or beak, having 3 to 4 segments, has its edges curved to form a groove. Within this groove are the biting parts—the bristle-like mandibles and maxillae.

The former are doubly grooved on their internal surfaces and thus when apposed form two tubes, one for injection of saliva and the other for suction of juice or blood.

The maxillae support the mandibles. When in repose the beak or rostrum is bent back under the head or thorax. The beak is covered by the labrum only at its base, thus differing from the Diptera in which the labrum goes into formation of the sucking tube.

Bugs have no palpi. They have two pairs of wings which in some genera, however, are rudimentary. The metamorphosis in this order is not marked.

Cimicidae

These have a flattened body, a three-jointed rostrum, and four-jointed antennae. Their wings are atrophied.

Cimex lectularius (**Acanthia lectularia**).—This is the cosmopolitan bedbug or chinch. It measures about 1/5 by 1/8 inch (5 by 3 mm.). It is of a brownish-red color. The most conspicuous feature of the bedbug is the long proboscis continuous with the dorsal integument of the head and tucked under the ventral surface. In biting the proboscis is straightened out and 4 piercing stylets are protruded to puncture the skin. There are two prominent eyes and two four-jointed antennae. The prothorax is flattened at the side. There are eight abdominal segments. The bedbug lives in cracks and crevices, especially about beds. It is said they can migrate from house to house. It is certain that they are frequently transferred with wash clothes. They have a penetrating odor when crushed. The female deposits about 50 eggs at a time in cracks and in ten days they hatch out into larvae which pass insensibly into adults by a series of five moultings during a period of 2 or 3 months. The depositing of eggs occurs about four times a year.

The bedbug has been considered as the intermediate host in kala-azar and it has been incriminated in connection with typhus fever and relapsing fever. It is known to be capable of transmitting plague.

Cimex rotundatus (**A. rotundata**).—In India the *C. rotundatus* is the one encountered. It is of a dark mahogany color, has a smaller head, narrower abdomen, thick rounded prothoracic borders and is more densely covered with hairs than *C. lectularius*.

Reduviidae

These bugs have a long narrow head and a distinct neck. The antennae are long and slender. The antennae in the genus *Triatoma* are inserted about midway between the eyes and point of the head.

Triatoma sanguisuga (**Conorhinus sanguisugus**).—This is known as the Texas or Mexican bedbug, and was formerly the foe of the common bedbug, but having gotten a taste for human blood through the *Cimex* or *Acanthia*, it now prefers man. It is extending toward the North. It has wings. The bites are much more severe than those of the common bedbug. It is of a dark-brown color, nearly an inch in length, with a long, flat, narrow head and a short thick rostrum. They can run as well as fly. They bite at night.

Triatoma megista (**Conorhinus megistus**).—This is called "Barbeiro" in Brazil on account of its preference for biting the face. The *Schizotrypanum cruzi* undergoes

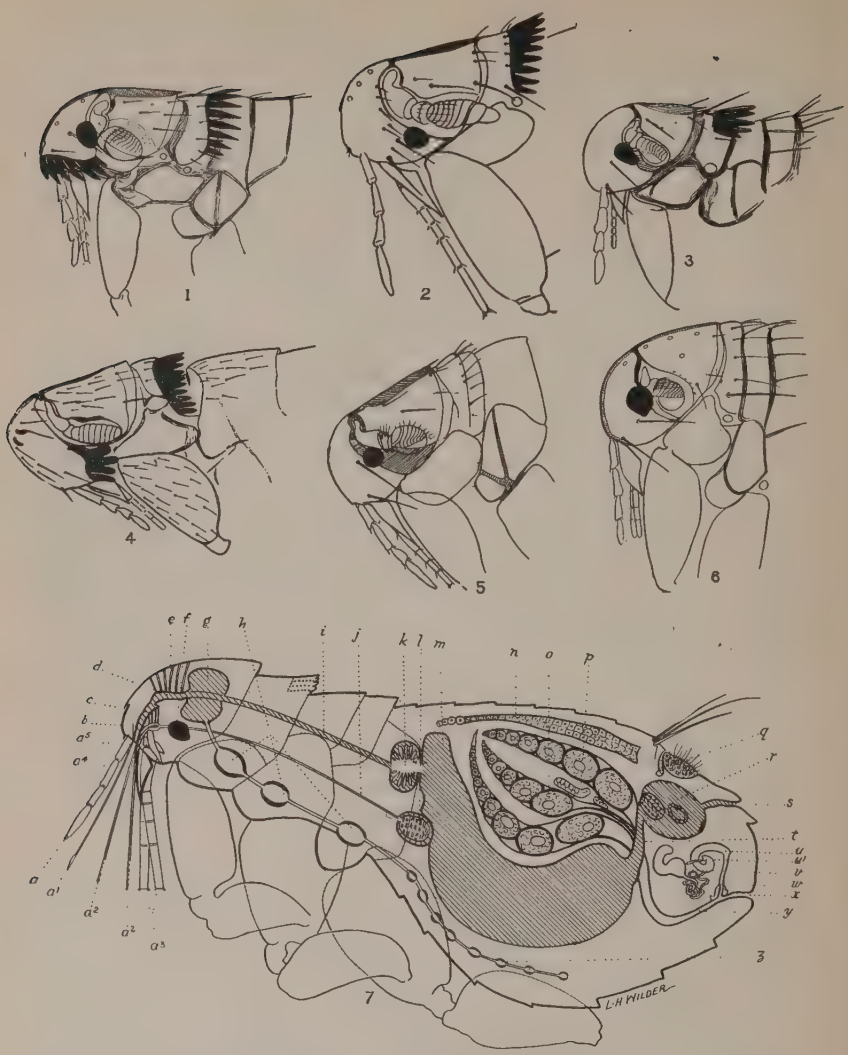


FIG. 156.—1. *Ctenocephalus felis.* 2. *Ceratophyllus fasciatus.* 3. *Hoplopsyllus anomalus.* 4. *Leptopsylla musculi.* 5. *Xenopsylla cheopis.* 6. *Pulex irritans.* 7. Internal anatomy of flea. (*After Fox.*) (*a*) Maxillary palpus; (*a*-1) epipharynx; (*a*-2) mandible; (*a*-3) labial palpi; (*a*-4) maxillae; (*a*-5) basal elements of rostrum and mandibles; (*b*) salivary pump; (*c*) hypopharynx; (*d*) aspiratory pharynx; (*e*) muscles of the aspiratory pharynx; (*f*) eye; (*g*) oesophageal ganglia (brain); (*h*) thoracic ganglia; (*i*) oesophagus; (*j*) salivary duct; (*k*) gizzard; (*l*) salivary gland; (*m*) stomach; (*n*) aorta; (*o*) ovaries; (*p*) Malpighian tubules; (*q*) pygidium; (*r*) rectum showing rectal glands; (*s*) anus; (*t*) intestines; (*u*) bursa copulatrix; (*u*-1) ductus obturatorius (blind duct); (*v*) receptaculum seminis or spermatheca; (*w*) ducts of spermatheca; (*x*) vagina; (*y*) uterus; (*z*) abdominal ganglia.

a developmental cycle in this bug and is transmitted by it. (See p. 409.) The bug is black with red markings on wings, abdomen and prothorax. The larvae hatch out of the eggs, which are deposited in the house, in about one month and a period approximating a year is required before the adult stage is reached. The wingless larvae crawl out of the cracks to feed on the sleeping inmates of the house. It has been suggested that the armadillo may be the animal preferred by these bugs and that it may be an important reservoir of virus for *Schizotrypanum cruzi*.

Rhodnius prolixus.—This bug is common in Venezuela and Brumpt has shown that it is capable of transmitting *Schizotrypanum cruzi*. Its bite is very painful. The basal joints of the antennae are inserted near the extremity of the head.

Siphonaptera

These are laterally flattened, markedly chitinized, wingless insects which undergo a complete metamorphosis.

This order is divided by Dalla Torre into two sub-orders—(1) the Fracticipita which contains the family Histrichopsyllidae of which the genus *Leptopsylla* is of medical interest and (2) the Integricipita containing the following families and genera of medical interest. Pulicidae—genera *Pulex* and *Xenopsylla;* Archaropsyllidae—genus *Ctenocephalus;* Dolichopsyllidae—genera *Ceratophyllus* and *Hoplopsyllus;* Tungidae—genus *Tunga*.

In the Tungidae family, which will be discussed separately, the abdomen of the female becomes enormously distended with eggs and she remains fixed in the burrow she has made under the skin, whereas, in all other families the female remains practically unchanged with freedom of movement after fecundation.

With the exception of infection with *Dipylidium caninum*, the fleas were for some time under suspicion as carriers of disease, ideas having been entertained as to their being possible transmitters of relapsing fever, typhus fever and kala-azar. As a result of the convincing experiments of the Indian Plague Commission, their rôle in the transmission of plague was absolutely established. It is by the bite of *Xenopsylla cheopis* (*X. pallida?*) that plague is chiefly transmitted from rat to rat, and in bubonic and septicaemic plague it is apparently the intermediary in human infection. Any species of flea which lives on the rat is capable of transmitting plague as would also be the case if *Pulex irritans* fed on the blood of a human case of septicaemic plague. *Trypanosoma lewisi* also is transmitted by fleas, either *Pulex irritans* or *Ctenocephalus canis*. The trypanosome undergoes development in the flea and the infecting material is in the faeces of the flea and transmission occurs by the licking on the part of the rat of faeces from an infected flea. The infection has no connection with the puncture wound of the flea as is the case with plague.

The average capacity of a flea's stomach is about 0.5 cu. mm. so that with a rat dying with septicaemic plague and with possibly 100,000,000 bacilli to 1 cc. of blood the flea would take in about 5000 bacilli. Furthermore these multiply in

the alimentary canal so that the digested blood teems with bacilli when reaching the anus of the flea. The plague bacilli are passed out with the faeces and these being rubbed into the puncture of the flea bite bring about infection. Regurgitation as result of obstruction by masses of plague bacilli in the oesophagus causes injection of plague bacilli into rat or man in the act of biting. This is more important than the faeces inoculation method. The puncturing apparatus of the flea consists of a pointed epipharynx and two distally serrated mandibles. These chitinous biting parts are contained in the labium which divides distally into two labial palps. The maxillae are conspicuous triangular structures and, projecting farthest anteriorly, are the conspicuous four-jointed maxillary palps, often mistaken for antennae. By the apposition of the internally grooved mandibles to the epipharynx a tube is formed through which the blood is sucked up. The antennae are inconspicuous and are in close apposition to the sides of the head, behind the eyes, and can only be well made out with a lens. Fleas have three pairs of legs and the male can be distinguished from the female by its smaller size and the conspicuous coiled-up spring of penis within the abdomen. The female has a conspicuous gourd-like spermatheca which varies in shape in different species. A very prominent structure is a pitted plate in the ninth abdominal segment (pygidium). Of importance in classification are prominent bristles originating from the seventh abdominal segment and projecting over the pygidium. These bristles vary in number and are known as antipygidial bristles.

The body of the flea is flattened laterally. They may or may not have eyes and certain conspicuous structures called combs which are of importance in classification. In the metamorphosis of the flea a bristled worm-like larva emerges from the egg in 3 or 4 days. These eggs fall from the hairs of the host on the dust of the floors. The larva has 14 segments and a distinct head which carries biting mandibles. These larvae feed on organic matter surrounding them and in this way probably ingest the eggs of *Dipylidium caninum*, the larval stage of which develops in the adult flea. Should such an infected flea be taken into the mouth of a dog or child the tape-worm infection results. The larva forms a cocoon and develops into a nymph which has three pairs of legs. The nymphs emerge from the cocoon as adult fleas in about three weeks after the larva forms it.

Key to Fleas Commonly Found on Rats and California Ground Squirrels

A. With combs.
  1. Eyes present.
    (*a*) Combs along inferior border of head and on prothorax. *Ctenocephalus canis* and *C. felis*.
    (*b*) Combs only on prothorax.
      (1) Rostrum extending to trochanters.
        Prothoracic comb of about 18 spines.
        Ceratophyllus fasciatus.
      (2) Rostrum extending well beyond the trochanters.
        Prothoracic comb of about 18 spines.
        Ceratophyllus acutus.

(3) Rostrum scarcely reaching half the distance to the anterior coxae. Prothoracic comb of about 9 spines.
Hoplopsyllus anomalus.

2. Eyes absent.
(*a*) Collar of combs on prothorax and four short ones along inferior border of head. *Leptopsylla musculi* (*Ctenopsylla musculi*).

B. Without combs.
(*a*) Ocular bristle arises near upper anterior margin of eye. A line between this and the oral bristle approximately vertical. Two bristles posterior to antennae. *Xenopsylla cheopis* (*Xenopsylla pallida?*). Formerly *Pulex cheopis.*
(*b*) Ocular bristle arises near lower anterior margin of eye. A line between this and the oral bristle approximately horizontal. One bristle posterior to antennae. *Pulex irritans.*

The common human flea of Europe is the *Pulex irritans;* those of the United States the *Ctenocephalus canis* or dog flea and *C. felis* or cat flea. The flea that is prominently implicated with plague is the *Xenopsylla cheopis*, on account of its being the common rat flea of India, where it has been much studied. It resembles *P. irritans*, but is more yellow than brown in color. It also has a greater number of bristles on the head. The ocular bristle runs above and in front of the eye; that of *P. irritans* below. It is principally the flea of *Rattus decumanus* (*R. norvegicus*), the sewer rat; but the house rat, *R. rattus*, becomes infected from coming in contact with the sewer rat in the basement. In the U. S. the ground squirrel, *Citellus beechyi*, acts as a reservoir of plague and has as its fleas *Hoplopsyllus anomalus* and *Ceratophyllus acutus.*

Ceratophyllus fasciatus is the common rat flea of Europe and the U. S. In the tropics *X. cheopis* is the common rat flea (98% in India). *Ctenocephalus canis* and *felis*, *Leptopsylla musculi* and *Pulex irritans* have also been frequently found on both *Rattus norvegicus* and *R. rattus.*

Rat Hosts.—To distinguish *R. norvegicus* from *R. rattus* we have in the former (1) ears which barely reach the eyes when laid forward and (2) tail rather shorter than length of head and body together (only 89% of length of head and body together). With *R. rattus* the tail is longer than the head and body together (25% longer) and the extended ear covers or reaches beyond the middle of the eye. *R. rattus* has a sharper nose, longer and more delicate tail and thinner ears than *R. norvegicus* (formerly *R. decumanus*).

R. alexandrinus is a variety of *R. rattus.* Rats and mice belong to the family Muridae and the common mouse is *Mus musculus.* They belong to the order of Rodentia of the class Mammalia.

Tungidae.—Belonging to the family Tungidae, the *Tunga penetrans* (*Dermatophilus penetrans*) is of great importance in tropical countries. It is known as the chigoe, nigua, or jigger. The male and virgin female are relatively unimportant as they do not penetrate the skin but act as ordinary fleas. The female, which when unimpregnated is only about 1/24 inch long, when impregnated bores its way into the skin of man, especially about the toes, soles of the feet or finger-nails, and in the chosen site develops enormously, becoming as large as a small pea. This enlargement takes place in the second and third abdominal segments which are packed with

eggs measuring about 400 microns long and numbering about 100. Clinically, a small black spot in the center of a tense rather pale area is characteristic. The metamorphosis is similar to that of the flea. *Tunga* can be differentiated from the

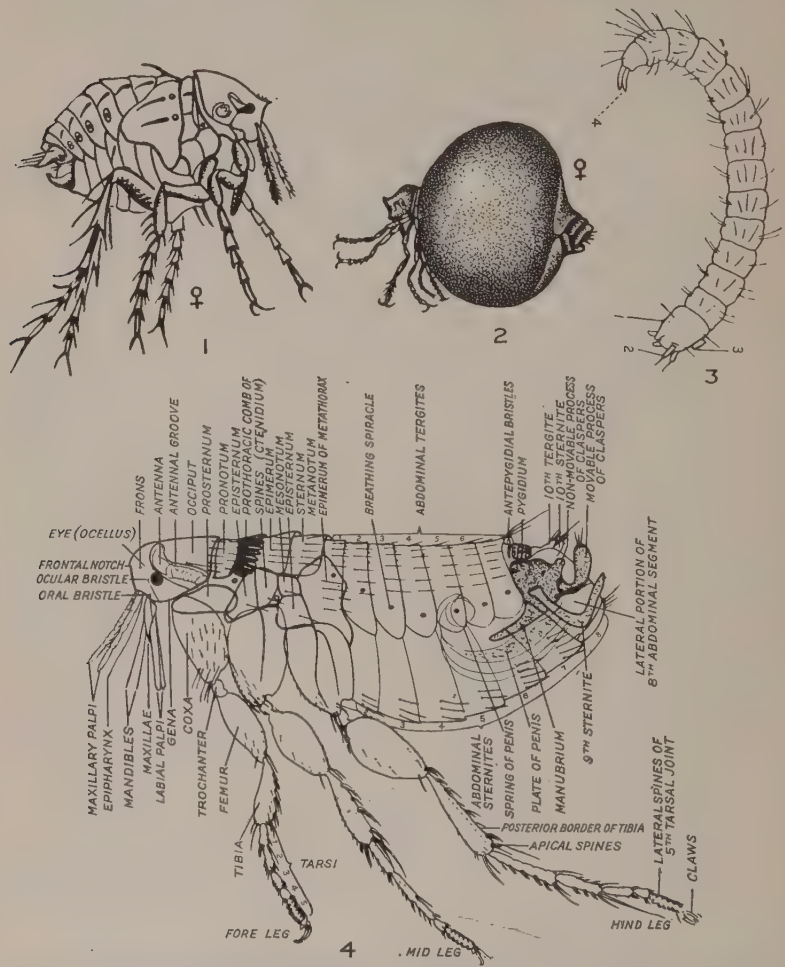


FIG. 157.—1–2. *Tunga penetrans*. (1) Male; (2) egg-distended female. 3. Flea larva highly magnified. (1) Head; (2) antennae; (3) egg-breaker; (4) caudal stylets. (*From Byam and Archibald.*) 4. External anatomy of a flea. (*After Fox.*)

flea by the proportionately larger head, and especially by the fact that the head has the shape of the head of a fish, distinctly pointed. With the fleas the lower border of the head comes out in a straight line to join the curve of the upper part. In the *Tunga* lower and upper border of head are both curved.

Diptera

The insects of the order Diptera are of great importance medically in a variety of ways, either by the direct irritation of their bites, by their transmitting disease directly, as does the common house fly typhoid fever, or by acting as intermediate or definitive hosts for various parasites. They are characterized by mouth parts formed for puncturing, sucking, or licking. They present a complete metamorphosis, larva, pupa, and imago. As a rule, the Diptera have a distinct pair of wings, the second pair being rudimentary (halteres).

The order Diptera is usually divided into the following suborders: (1) *Orthorrhapha:* Diptera with larvae having a differentiated head. The imago breaks through the larval or pupal case by a T-shaped break on dorsum and has no frontal lunule (an oval space just above the root of the antennae). The Orthorrhapha are divided

Fig. 158.—*Chrysops discalis*, showing the characteristic nonpigmented discal cell whence is derived its name.

into: (*a*) Nematocera (with long, many-jointed antennae), and (*b*) Brachycera (with short antennae).

The Nematocera are generally midge-like insects and have, as a rule, long slender palps (mosquito). The Brachycera, however, are seldom midge-like. The antennae are composed of only two or three simple joints with or without style or arista. The palps are almost always short and never more than two-jointed. (2) *Cyclorrhapha:* Larvae without differentiated head. The imago escapes through an anterior circular opening in the puparium, produced by the ptilinum. This is an inflatable organ projecting just above the root of the antennae and is withdrawn as soon as imago has escaped, leaving behind the typical scars, the frontal lunule and the frontal or ptilinal suture (see Fig. 159).

The suborder Cyclorrhapha is divided into the following sections. (*a*) Section Aschiza with a small ptilinum hence the insignificant scar at base of antennae (Syrphidae, Phoridae). (*b*) Section Schizophora with a large ptilinum and hence the marked frontal lunule and sutures causing retraction of front of head. Head is freely movable. If the halteres are covered by a scale (squama) we have calyptrate Schizophora; if not, acalyptrate. These squamae are large enough in the calyptrate species even to conceal the halteres when the fly is looked at from above. (3) *Pupipara:* The larvae are extruded from the mother and almost immediately begin the pupal life. Leathery flies with poorly developed wings (Hippoboscidae).

The males of flies where the two compound eyes come together above the antennae are referred to as holoptic, if more or less widely separated as dichoptic. Ocelli are single eyes, usually three in number, and, when present, situated in the triangular space between the compound eyes in the frons (the space separating the compound eyes).

The anterior portion of the head which lies below the origin of the antennae is the face and on each side of the face we have the cheeks which should be studied as to presence or abundance of hairs. The antennae which separate the frons from the face are of great importance in classification. In the Muscidae the appearance of a feathery structure, projecting from the terminal segment of the antennae, and called the *arista*, is important. This may be bare or feathered and the feathering may be only on one side or of one part.

In studying the biting flies it is very important to recognize the anterior, small, or mid-cross vein in the wings. This short transverse rib or vein is the key to wing venation. Beneath it is the discal cell and it bounds the first posterior cell internally or basally. The fourth longitudinal vein, which touches the bottom of the mid-cross vein, is of particular importance as it gives different shapes to the first posterior cell as it runs along the lower border of this cell. The closed-in discal cell is below the fourth longitudinal vein. The character of the antennae should also be noted carefully. The study of the bristles about head, thorax, and abdomen (chaetotaxy) is more difficult. Anyone taking up the study of flies should carefully note the wings, etc., of *Musca domestica*. By putting a few house flies on moist horse manure in a gauze-covered bottle the entire metamorphosis may be observed.

Tabanidae

This is the family of horseflies, gadflies, breeze flies or green-headed flies. It is the most numerous family of the Diptera—there being nearly 2000 species. The females are blood-suckers; the males live on flowers and plant juices. The eyes are usually very brilliant in color, and in the male make up the greater part of the head.

They belong to the suborder Orthorrhapha and in the group of short-antennae flies (Brachycera). The wings are large and encircled by the costal vein. The third longitudinal vein is forked. The fourth longitudinal vein breaks up three times

thus enclosing the discal cell. Five posterior cells are always present. The squamae are large.

The antennae consist of three segments, the third of which is compound. No arista. The mouth parts are complete in the female. The epipharynx is tube-like, the hypopharynx has a groove and both are awl-shaped. The paired maxillae

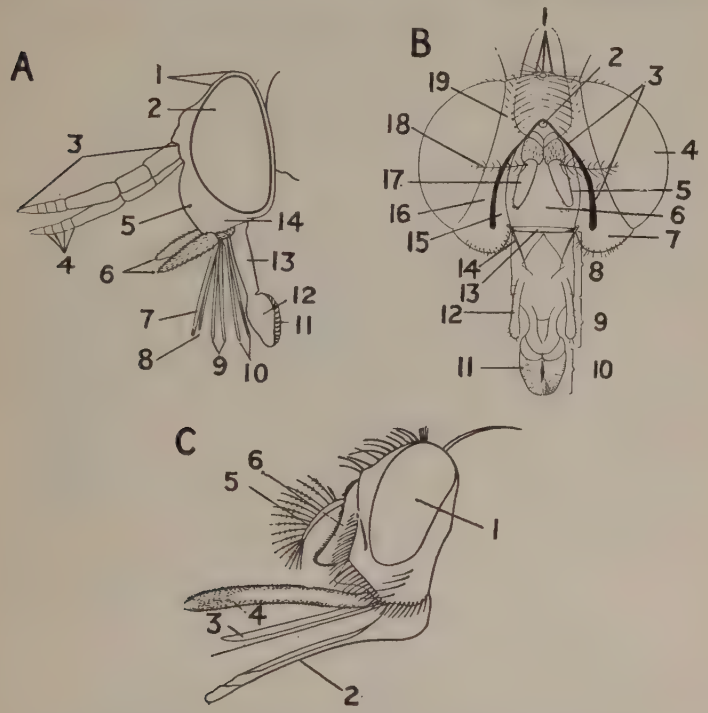


FIG. 159.—Heads of flies (semidiagrammatic).

A, *Chrysops discalis*. (1) Ocelli; (2) compound eye; (3) antennae; (4) annuli; (5) clypeus; (6) palpi; (7) labrum-epipharynx; (8) hypopharynx; (9) mandibles; (10) maxillae; (11) pseudotracheal membrane; (12) labellae; (13) labium; (14) gena.

B, *Muscoidean fly*. (1) Ocelli; (2) lunula; (3) ptilinal suture; (4) compound eyes; (5) antennal grooves; (6) clypeus; (7) genae or cheeks; (8) rostrum; (9) haustellum; (10) labellae; (11) pseudotracheal membrane; (12) palpi; (13) epistoma; (14) oral vibrissae; (15) facialia; (16) parafacials; (17) antennae; (18) arista; (19) parafrontals. (*After Fox.*)

C, *Glossina* sp. (1) Eye; (2) labium; (3) labrum; (4) palp; (5) arista; (6) antenna

are serrated and the mandibles lancet-like. They have rather coarse maxillary palps. The labellae are prominent at the extremity of the fleshy labium. In the male the mandibles are atrophied. The Tabanidae are thick-set flies and rarely show color. The body of the larva has eleven segments and a small but distinct

head. The eggs are deposited in masses on the leaves or stems of plants about marshy places. The larva is carnivorous.

Tabanus autumnalis.—It is about ¾ inch long; is dark in color, and has four longitudinal bands on the thorax. The last joint of the antennae has a crescentic notch and is composed of five parts. The wings do not overlap.

Haematopota pluvialis.—In the *Haematopota* there is no crescentic antennal notch, and the third antennal segment is composed of four parts. The wings overlap. The abdomen is narrower than in *Tabanus*. The brimp, one of the *Haematopota*, bites man severely.

Pangonius beckeri.—The genus *Pangonius* is characterized by a very long, slender and more or less horizontal proboscis. The antennae are small. The third segment is composed of seven or eight parts.

Chrysops discalis.—*Chrysops* has three ocelli, in this respect differing from the genera *Tabanus* and *Haematopota*. The wings are widely separated and spotted. The antennae of *Chrysops* are especially long and slender and have three segments, the last one of which is composed of five parts. *Chrysops* and *Haematopota* produce the greatest amount of pain from their bites. The Tabanidae except *Chrysops* are not implicated as intermediate hosts in the transmission of disease. By their bites, however, they may transmit disease directly, as with anthrax. Two species of *Chrysops, C. dimidiata* and *C. silacea*, transmit *Loa loa* and *C. discalis* the organism of tularaemia.

Muscoidea

Under this heading we may group the Muscidae, Sarcophagidae and Oestridae which are calyptrate Schizophora.

Muscidae

In the Muscidae the antennae hang down in front of the head in three segments and have an arista plumose to the tip on one or both sides. The first posterior cell is narrowed due to bending up of 4th vein. There are no bristles on abdomen except at tip.

Adult Muscid Flies That do not Suck Blood.—*Musca, Calliphora, Chrysomyia, Lucilia, Auchmeromyia* and *Cordylobia* do not have a proboscis adapted for biting. The larva of *Auchmeromyia*, however, is a blood-sucker and the larvae of *Chrysomyia* bore their way into tissues.

Musca domestica.—The common housefly, *Musca domestica*, is the best example of this family.

The arista is feathered both dorsally and ventrally with straight hairs. The fourth longitudinal vein bends forward in a rather sharp angle as compared with *Stomoxys*, the first posterior cell of the latter having rather a fusiform appearance. The eyes are close together in the male, far apart in the female. In contrast to the other flies in this group *musca* has no large bristle on the inner surface of tibia

of the middle legs. The female lays about 125 eggs in a heap preferably in fermenting horse manure. The larva comes out in about thirty-six hours. Very characteristic are the stigmata decorating the blunt posterior ends. (See illustration.)

The larval stage lasts seven to ten days and then the larva shrinks but remains surrounded by its old skin, termed puparium, which forms the covering for the barrel-shaped pupal stage. This lasts about three days when the adult fly emerges. This is termed a "coarctate" pupa. This fly is incapable of biting, the piercing organs being fused with the labium, but may transmit disease directly, carrying infectious material from the source, as faeces, to the food about to be ingested. Their rôle in typhoid fever is one of importance. By reason of its hairy sticky legs, habits of frequent defaecation and constant regurgitation the housefly is an impor-

FIG. 160.—Common housefly (*Musca domestica*): Puparium at left; adult next, larva and enlarged parts at right. All enlarged. (*From circular 71 (by L. O. Howard), Bureau of Entomology, U. S. Department of Agriculture.*)

tant agent in the spread of cholera, dysentery, infantile diarrhoeas and tropical ophthalmias as well as typhoid.

Auchmeromyia luteola.—This, an African fly, the larva of which is known as the "Congo floor maggot," is a blood-sucker. The larva is of a dirty-white color and about ⅔ inch long. It crawls out at night and feeds on the sleeping native. This is the only known instance of a blood-sucking larva. The adult fly resembles the blow fly but is yellowish-brown. The fly deposits her eggs on the floor of native huts by preference. The larvae hatch from the eggs in a few days and seek blood soon afterwards. These larvae are much like those of *M. domestica.*

Calliphora vomitoria and Lucilia caesar.—*C. vomitoria* is a large bloated fly of bluish color with slight metallic lustre. It is the common blow fly, also called blue-bottle fly. The cheeks are hairy. *L. caesar* is a smaller fly of shiny, metallic green color with a bluish tinge. It is called the green-bottle fly. The cheeks of this genus are bare. These flies deposit their eggs on tainted meat and in wounds. Many cases of obscure abdominal trouble are probably due to the larvae of these flies. Intestinal myiasis is undoubtedly of greater importance than has been

thought. The larvae, with hook-like projections anteriorly and a ringed body, can easily be recognized in the faeces. They have been mistaken for flukes. They also have a tendency to be attracted by those with ozaena and the larvae may develop in the nostrils.

Chrysomyia macellaria.—This is known as the screw-worm when in the larval stage. The adult resembles the green-bottle flies but is somewhat larger. It is distinguished from them, however, by the presence of black stripes on thorax. These

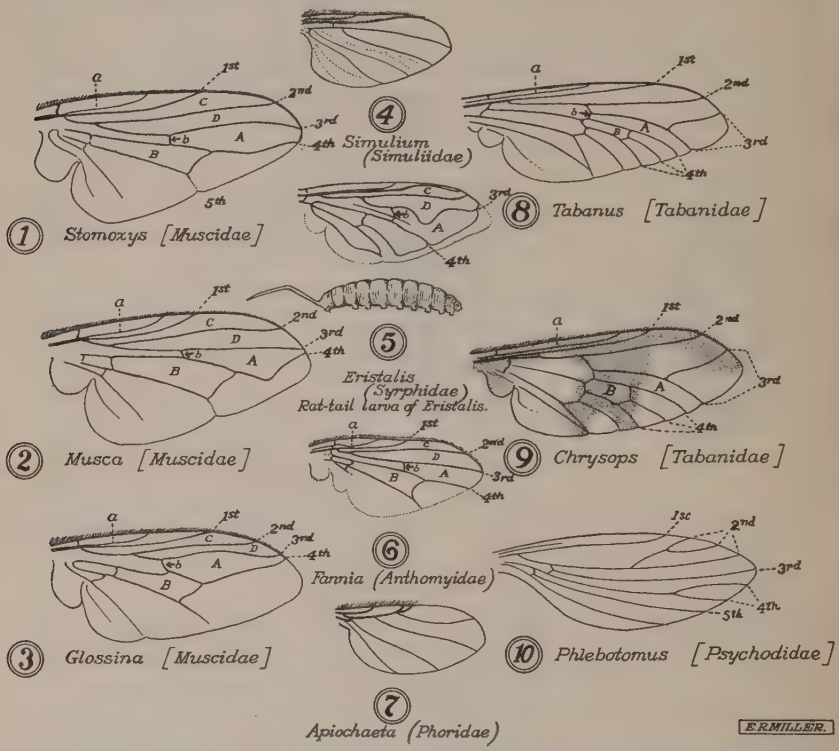


FIG. 161.—Wing venation of Diptera. *A*, First posterior cell; *B*, discal cell; *b*, mid-cross vein; *a*, auxiliary vein; *C*, marginal cell; *D*, submarginal cell.

flies are very common over nearly all North and South America. The eggs, which number 250 or more, when deposited in the nostrils or in wounds, develop into the screw-worm larvae which may, by going up into the frontal sinus, cause death. These larvae have twelve segments with rings of minute spines. They reach maturity in 3 or 4 days when they are about ½ inch long.

Cordylobia anthropophaga (*Ochromyia anthropophaga or* Tumbu Fly).—This is an African fly whose larvae develop under the skin of man and animals. It is known as the Ver du Cayor. The larva resembles the Ver Macaque, is rather

barrel-shaped and beset with small spines. It bores its way into the skin and makes a lesion like a boil which has a central opening through which the larva breathes. The fly deposits its eggs, which contain larvae ready to emerge, on clothing or about the head of very young children. These larvae also infest other animals especially young dogs. They apparently bore into the skin painlessly and a single child may harbor 15 or 20 of these maggots. The larva reaches maturity, when it is about ½ inch long, in 12 to 14 days.

Blood-sucking Muscid Flies.—*Stomoxys*, *Haematobia* and *Glossina* have a more or less elongated proboscis adapted for biting. *Stomoxys* has delicate palpi, shorter than the proboscis, and arista feathered only on the dorsal side with straight hairs. *Haematobia* has club-like palpi about as long as proboscis, and arista with hairs dorsally and ventrally. *Glossina* has thick-set but not clubbed palpi as long as the proboscis for which they serve as a sheath. The arista is feathered on the dorsal side with branching hairs.

Stomoxys calcitrans.—The stable fly greatly resembles the common housefly in size and shape. It can be easily distinguished by the black, piercing proboscis extending beyond the head. There are longitudinal stripes on the thorax and spots on the abdomen. The proboscis on examination will be seen to be bent at an angle near its base. The palps are short and slender. The wings diverge widely. The 4th longitudinal vein has a gentle forward curve.

The female lays about 60 banana-shaped eggs in horse manure. These hatch out in three days as larvae which turn into pupae in two or three weeks. After about ten days the fly emerges. The genus *Stomoxys* includes vicious biters. This is the fly which comes into houses before a rain, and which has given the common housefly the reputation of biting before a rain. *Stomoxys* may be implicated in transmitting surra (*Trypanosoma evansi*).

It assumed great importance as a transmitter of poliomyelitis and possibly of pellagra a few years ago—views now discredited.

The horn fly (*Haematobia irritans*) rarely bites man. In this the palpi are much longer than in *Stomoxys*, being as long as proboscis. These palps are also thick and spatulate.

Glossina palpalis.—This is the tsetse fly that is responsible for the transmission of human trypanosomiasis (sleeping sickness).

The tsetse fly is a small brownish fly about ⅓ inch long. The proboscis extends horizontally and has a bulb at its base. The arista is plumose only on the upper side and the individual hairs are themselves feathered. The wings are carried flat, closed over one another like the blades of a pair of scissors and project beyond the abdomen. The most characteristic feature of the tsetse fly is the way the fourth longitudinal vein bends up abruptly to meet the mid-cross vein and then curves

downward to run parallel with the third longitudinal vein before it turns forward again to end at the anterior border of the wing. Glossinae bite chiefly in the daytime.

The tsetse fly is much like *Stomoxys*, but has a branching of the feathering of the arista, long palps, a bulb to the proboscis and a characteristic upbending of the fourth longitudinal vein to meet the mid-cross vein. The female deposits her larva near a shady place upon loose, dry, sandy soil. Moisture and sunlight are not favorable for pupal development, the sun being particularly injurious, so that pupae, buried only an inch deep and away from shade, are killed. This fact has been utilized in attempted eradication of the disease, trees being cut down. The trouble is that the bush growth which soon follows is favorable as shade for the pupae.

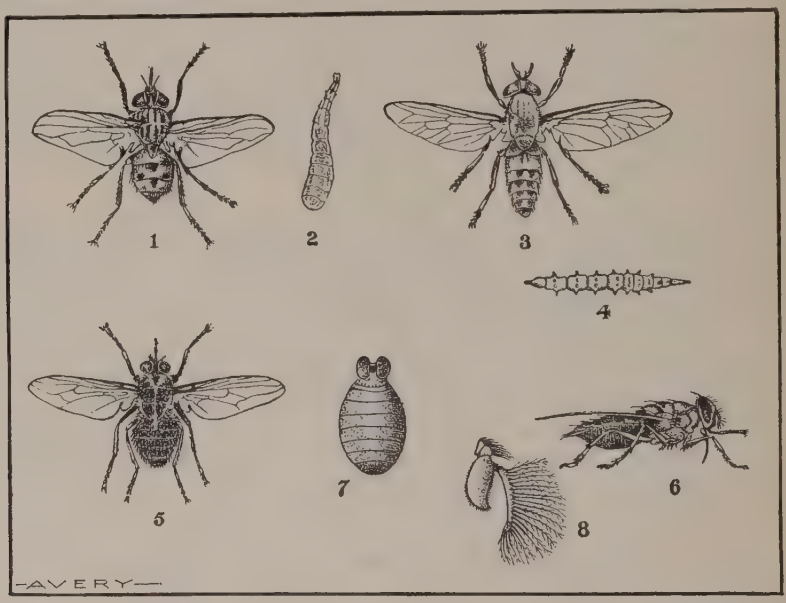


FIG. 162.—Insects in which the adult stage is important. (1) *Stomoxys calcitrans;* (2) *S. calcitrans,* larva; (3) *Tabanus bovinus;* (4) *Tabanus* larva; (5) *Glossina palpalis;* (6) *G. palpalis,* side view; (7) *G. palpalis* pupa; (8) *Glossina* palps and arista.

The female gives birth to a single, yellowish-brown, motile larva, which is almost as large as the mother and which, upon reaching the ground, bores its way into a coarse, sandy soil for a depth of about 2 inches and then becomes a pupa. The larval stage in the mother lasts about two weeks and the pupal stage in the ground about a month.

Both male and female bite and transmit the disease. They bite in the daytime, usually from 9 A. M. to 4 P. M., and will bite in the sunlight.

With a view to eradication of the disease certain areas have been depopulated, but upon examining the flies caught in the district a year or more later, infected flies have been obtained. This would indicate some other reservoir than man. It is

now generally conceded that the trypanosome strain in the antelope is the same as *T. rhodesiense*, both being transmitted by *G. morsitans*. Taute however believes them different as he not only injected blood containing such trypanosomes into himself, with negative result, but also allowed flies which had fed on antelopes, which were infective for laboratory animals, to feed on himself, likewise with negative result. It is a well known fact that men in good condition are refractory to trypanosome infection so that this courageous experiment does not prove the antelope strain to be different from the human one.

FIG. 163.—*Glossina palpalis* Rob. Des. Male × 4. (*After Austen; from Mense.*)

One measure that has been proposed is to kill off the big game from a certain area with a view to depriving the flies of their main source of infection.

The probabilities of an animal reservoir for *T. gambiense* however is not so well settled. Many think that we may have trypanosome carriers and that such persons while in the enjoyment of health may act as reservoirs of the virus. Koch suggested that crocodiles were important factors in the life of the tsetse flies and recommended the destruction of the crocodile eggs.

Glossina morsitans transmits the cattle trypanosome disease, nagana, and the human infection due to *Trypanosoma rhodesiense.*

Sarcophagidae

These are known as "flesh flies." The most important characteristic is the fact that the arista is plumose up to the mid-point, beyond which it is bare. They are usually thick-set and moderately large flies.

Sarcophaga carnaria.—This is a grayish fly with three stripes on thorax and black spots on each segment of the abdomen. It is viviparous. The larvae gain

access to nasal and other cavities and there develop. Cases of death have been reported. Naturally, the fly deposits its larvae on decaying flesh. In times of war all of these flies become important by reason of deposition of "maggots" in wounds. These larvae are the most common ones in intestinal myiases. The mouth hooklets are strongly curved and separate. Each abdominal segment has a girdle of spines. The anterior end is somewhat pointed. The hind stigmal plate is in a deep cavity.

Sarcophaga haemorrhoidalis is one of the most widely distributed species of this genus. Aldrich states that this is the only species that has as yet been proven to develop almost or quite to full size within the alimentary tract of man. He has reported several cases of severe intestinal myiasis in the United States due to this species.

Oestridae

The flies of this family are usually called warble or botflies. The mouth parts are almost vestigial. They have a large head with a somewhat bloated-looking lower portion. They are often rather hairy. The larvae which develop from the eggs are parasitic either in the alimentary canal or the subcutaneous tissues.

Dermatobia hominis (D. cyaniventris).—These are large, thick-set flies about ⅗ inch long, with prominent head and eyes, small antennae, and a marked narrowing at the junction of thorax and abdomen. The thorax is grayish and the abdomen a metallic blue. The larvae are deposited under the skin in various parts of the body. When the larvae move they cause considerable pain. At first the larva is club-shaped, but later on it becomes oval. The former is called ver macaque, the latter torcel.

Hypoderma diana.—The larval form of this fly has been reported three times for man. It forms tumors under the skin which it is thought may reach this location by proceeding in some way from the alimentary canal.

In *Hypoderma* the arista is bare while in *Dermatobia* the upper border is plumose.

Cutaneous Myiases

Ver Macaque.—The best known of these myiases is that due to the larva of a botfly, *Dermatobia hominis.*

The larva is at first club-shaped and in this stage is called ver macaque. Later on it becomes worm-shaped and is then called torcel in Venezuela or berne in Brazil. The natives of most of the countries where the infection is found have called the larvae "mosquito worms" or "gusano de zancudo" and they have even incriminated large mosquitoes belonging to the genus *Psorophora* as being responsible for the infections.

Surcouf has noted that these fly larvae have been found cemented to mosquitoes of the genus *Psorophora* by a glue-like substance. These mosquitoes are vicious biters and evidently the young larvae escape from the eggs attached to the mosquito and enter the wound made by the biting parts of the mosquito. Some have thought that *D. hominis* deposits its eggs in a glue-like material on the leaves of plants and that they stick to mosquitoes flying about such plants. From the facts that these eggs apparently become attached only to this particular mosquito and further

that the eggs are attached in a constant manner with the hatching end outward it would seem that the mother fly must in some way seize the mosquito and deposit her eggs on it. As the larva grows in the subcutaneous tissues of man or other animals a tumor-like swelling develops with a central orifice, toward which the posterior extremity of the larva points and through which it takes air into its spiracles.

The swelling somewhat resembles a blind boil and may be as large as a pigeon's egg.

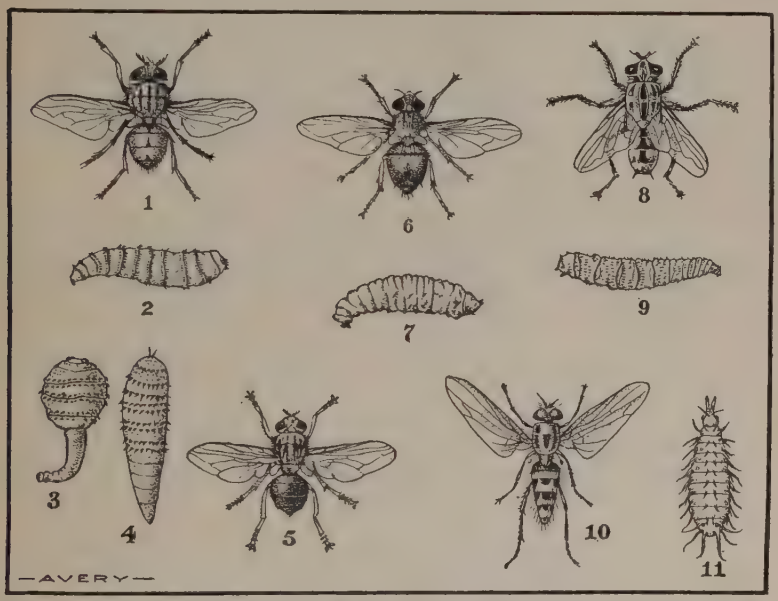


FIG. 164.—Insects in which the larval stage is important. (1) *Chrysomyia macellaria;* (2) *C.* larva; (3) *Dermatobia hominis* larva, early stage (ver macaque); (4) *D. hominis* larva, later stage (torcel or berne); (5) *D. hominis;* (6) *Auchmeromyia luteola;* (7) *A. luteola,* larva; (8) *Sarcophaga magnifica;* (9) *S. magnifica* larva; (10) *Anthomyia pluvialis;* (11) *A. pluvialis* larva.

These botfly boils tend to break down and discharge a sero-purulent fluid and it is supposed that the larva, when mature, escapes as a result of the disintegration of the tumor.

In Brazil they make tobacco juice applications which cause the larva to protrude and then squeeze it out. The injection of a little chloroform into the larva with a hypodermic syringe, prior to its extraction with a forceps, makes the process less painful.

The Screw-worm.—This is the larva of the *Chrysomyia macellaria.*

This muscid fly lays 200 to 300 eggs in wounds or orifices having offensive discharges, as nose, ears, etc. The larvae burrow into the adjacent tissues and cause

frightful destruction of all soft parts. The mature larvae are a little more than ⅔ inch long and have circlets of spines around each of the 12 segments.

This infection is especially common in tropical and subtropical America and is important in animals as well as man.

Ver du Cayor.—These maggots, which are the larvae of the *Cordylobia anthropophaga*, are widespread in Africa. They penetrate the skin especially of very young children who are not kept clean. The lesion rather resembles a boil.

Intestinal Myiases

In the tropics vague intestinal disturbances or violent abdominal cramping may be brought about by dipterous larvae in the intestinal canal. The symptoms may be those of a dysentery and may be attended with fever and malaise.

The larvae usually obtain access to the alimentary tract in food taken in by the mouth. Flies of the genus *Sarcophaga* are prone to deposit their larvae on food, especially meat that is somewhat tainted. Other flies, as *Musca* or *Anthomyia*, may lay their eggs on food. Flies of the genus *Anthomyia* tend to lay their eggs on plants.

It is possible for a fly to deposit its eggs or larvae about the anus while the man is at stool.

Great care must always be observed to assure one's self that fly larvae, which may be present in the stool, have not originated from larvae deposited on the stool subsequent to its passage.

Aural Myiases

While the larva of *Chrysomyia macellaria*, known as the "screw-worm," is the one most frequently reported from the external auditory canal yet many such cases have been connected with the larvae of *Sarcophaga carnaria*, *Calliphora vomitoria* and *Anthomyia pluvialis*. These larvae are usually deposited in the auditory canals of those with otorrhoea.

The symptoms are intense earache, giddiness and possibly convulsions. The larvae tend to perforate the tympanic membrane. Instillations of 10% chloroform in milk or the use of oils kill the larvae.

Determination of Dipterous Larvae

There are certain points in the anatomy of dipterous larvae which must be considered in determination of the genus or family of the flies concerned in the various myiases. The broad extremity is the posterior one and the tapering one the anterior. The dark hook-like processes,

which may be in pairs or fused, project from the anterior or head end and above them is a pair of projecting papillae. The second segment from the head has on either side projecting hand or fan-like structures with varying numbers of terminal divisions, 4 to 40 or more. These are the anterior spiracles.

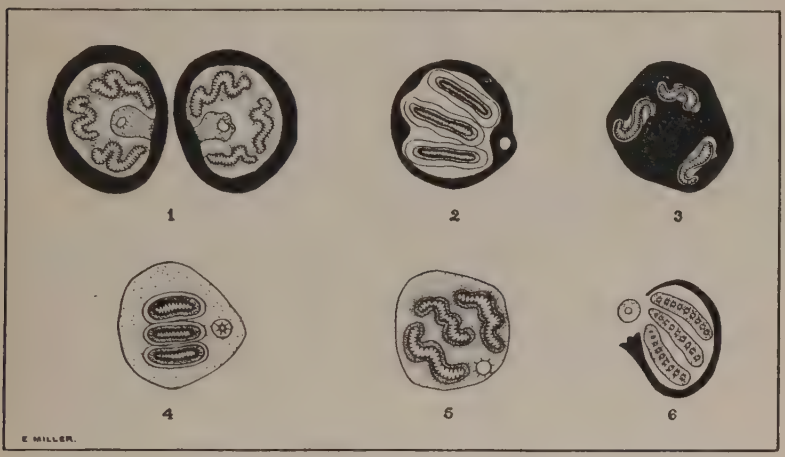


FIG. 165.—Markings of breathing slits on posterior stigmata of various dipterous larvae. 1. *Musca domestica,* showing both stigmata; 2. *Calliphora vomitoria;* 3. *Stomoxys calcitrans;* 4. *Auchmeromyia luteola;* 5. *Cordylobia anthropophaga;* 6. *Sarcophaga magnifica.*

The large terminal segment has on its posterior surface two chitinized plates with 3 slits of various architecture in each. These are the posterior stigmal plates and are the structures we pay particular attention to in identification. In the early larval stages there is only one slit; in the second stage there are two. It is only in the fully developed larval stage that we note the characteristic 3-slit stigmal plates. The presence or absence of a rounded protuberance or button at the base of each stigmal plate should be looked for. The area carrying the stigmal plates may be sunken to form a pit.

KEY TO THE LARVAE CAUSING MYIASES

(Adapted from Banks after Fox)

1. Body with spinous or fleshy processes laterally and dorsally or terminal..... 2
   Body without spinous or fleshy processes..... 3
2. Body with long lateral and dorsal spinous processes.....*Fannia* (*Homalomyia.*)
   Body with long tail-like process.....*Eristalis*
   Body ending in two small fleshy processes, rather small species, the processes bearing the stigmal plates, body about 5 mm. long.....*Drosophila*
   Same but processes not bearing the stigmal plates, body about 10 mm. long.....*Piophila*

3. Body robust, ovate, cylindrical, rounded at ends, slightly depressed, or body pyriform ..... Oestridae
   Body truncate broadly rounded at one end and tapering at the other (head) end. 4
4. But one great hook, posterior stigmal plates with winding slits; no distinct lateral fusiform areas, tip of body with few if any conical processes ..... Muscina (in part, including *Musca domestica*)
   With two great hooks; slits in the stigmal plates not sinuous 5
5. No tubercles about anal area; no distinct processes around stigmal field 6
   Distinct tubercles above anal area; often process around stigmal field; lateral fusiform areas usually distinct 7
6. Stigmal plates on black tubercles, lateral fusiform areas distinct ..... Ortalidae
   Stigmal plates barely if at all elevated; lateral fusiform area indistinct, stigmal plates often contiguous or nearly so; slits long and subparallel ..... Trypetidae
7. Slits in stigmal plates rather short and arranged radiately 8
   Slits slender and subparallel to each other 9
8. Two tubercles above anal area; stigmal field with distinct fleshy tubercles around it ..... Anthomyiidae (except *Fannia*)
   Four or more tubercles above anal area; slits of stigmal plates usually pointed at one end ..... Muscina
9. A button to each stigmal plate; slits rather transverse to body .... Calliphoridae
   No button to stigmal plate; slits of one plate subparallel to those in opposite plate; plates at bottom of a pit ..... Sarcophagidae

CHAPTER XXII

THE MOSQUITOES

Mosquitoes (Culicidae) are of the greatest importance medically, not only from their influence upon health in general by reason of interference with sleep and possibly from direct transmission of disease, but, more specifically, they are the only means by which it at present appears possible to bring about infection with such diseases as yellow fever, malaria, filariasis and dengue. In addition, many diseases of animals are transmitted by mosquitoes.

The Culicidae differ from all other Diptera in having scales on their wings and generally on head, thorax, or abdomen, and the subfamily Culicinae, the true mosquitoes, differ from the other culicids by having a long proboscis.

To identify a mosquito, note the presence of a long proboscis, examine a wing and note the scales; also note the presence of two distinct fork cells and, in addition, that the costal vein passes completely around the border of the wing, making a sort of fringe with its scales. Mosquitoes undergo a complete metamorphosis, there developing from the egg a voracious, rapidly growing larva, which moults four times, transforming to pupa with the fourth moult. The pupa or nymph constitutes a nongrowing or nonfeeding stage in which the head and thorax are combined in an oval body, from the back of which project the siphon tubes; and tucked in ventrally is a small tail-like appendage.

The fully developed insect emerges from the pupa.

The Culicidae belong to the suborder Orthorrhapha, section Nematocera, characterized by long articulated antennae. It includes four families: Culicidae, Chironomidae, Simuliidae and Psychodidae.

The principal mosquito-like, blood-sucking Diptera which are frequently mistaken for mosquitoes—but differ in not having scales on their wings—are the following:

1. **Chironomidae** or Midges.—The blood-sucking species of *Chironomidae*, which are found in most parts of the world, belong chiefly to the genus *Ceratopogon*. These midges are of very small size, about $\frac{1}{12}$ inch long, are able to get through netting and, usually being in swarms, are exceedingly troublesome. The antennae have thirteen joints and the wings are shorter than the abdomen and have longitudinal veins only. One of the midges, the "jejen" of Cuba, is a great scourge, its small

size enabling it to enter eyes and nostrils. The larva of *Chironomus* is a red worm-like creature; the pupa has a tufted head.

2. **Simuliidae** or Buffalo Gnats.—These are small blood-thirsty insects only about 1/8 inch in length. The thorax is humped, the legs are short and the proboscis short and inconspicuous. The antennae have 11 joints but are rather short. One species, the *S. damnosum*, known by the natives of Uganda as "Mbwa," is greatly dreaded, its bites causing swellings and sores.

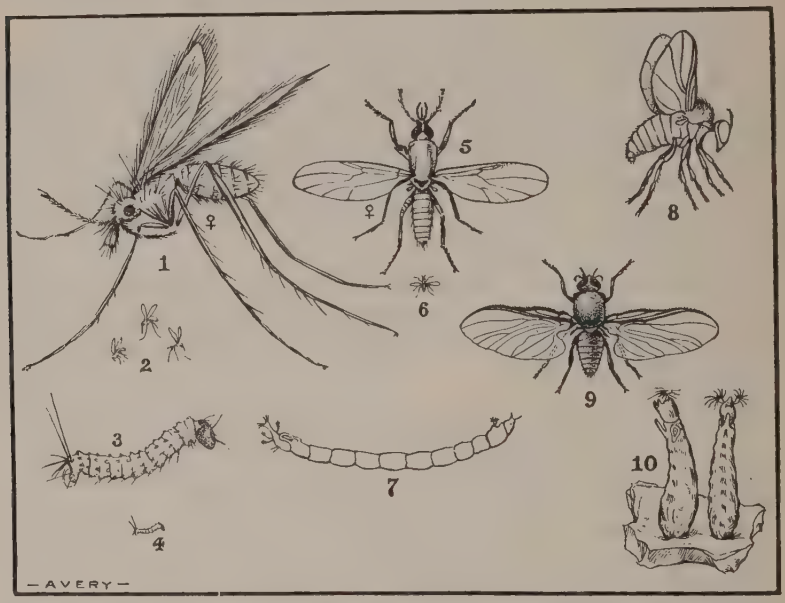


FIG. 166.—Mosquito-like insects belonging to families Chironomidae, Simuliidae and Psychodidae. (1) *Phlebotomus papatasii;* (2) *P. papatasii* (natural size); (3) *P. papatasii* (larva); (4) *P. papatasii* larva (natural size); (5) *Ceratopogon pulicaris;* (6) *C. pulicaris* (natural size); (7) *Chironomus* larva; (8) Attitude of a *Simulium;* (9) *Simulium reptans;* (10) Larvae of *Simulium.*

3. **Psychodidae** or Moth Midges.—These are small, hairy, slender midges, with long legs and a short proboscis. The antennae are long, hairy and consist of 12 to 16 joints. Palpi four-jointed. They are only about 1/12 inch in length. The hairy wings have numerous longitudinal veins. Some, as *Phlebotomus*, have an elongated proboscis and are vicious blood-suckers.

At present, of the genera of the three families of midges, only *Phlebotomus* is known to transmit disease. *P. papatasii* transmits phlebotomus fever in the Balkans. *P. minutus* is the host at Aden. Another species, *P. perniciosus*, can transmit the disease. Sergent, Adler, and others proved that the *Phlebotomus papatasii* can transmit leishmaniasis. These moth midges are 2 mm. in length and have the body densely covered with long yellow hairs. The second longitudinal

vein has three distinct branches. The antennae have 16 constricted joints and the proboscis is as long as the head. The species of *Phlebotomus* are differentiated by slight variations in wing venation, palpal lengths, etc., thus the second segment of palpi of *P. papatasii* is a little longer than the third one while with *P. perniciosus* these segments are of equal lengths. In *P. minutus* the second segment is only half the length of the third. The insect lays about 40 eggs in damp dark places. The period of metamorphosis from egg to insect is about one or two months, according to temperature.

Phlebotomus larvae die out in dry soil and very wet earth is unfavorable. Moderate moisture and protection from light seem necessary for their development. The remains of dead insects also seem to make good breeding places. It is in cracks of old damp brick or stone walls that the female most often deposits her eggs. Caves are also selected. Blood seems necessary for the fertilization of the eggs but lizard blood seems more common in the stomach of *P. minutus* than human blood. They have also been observed to feed on other reptilian bloods. The female insect has been kept alive in captivity up to forty-six days.

Townsend believes that verruga peruviana is transmitted by the bite of *P. verrucarum*, a midge which is abundant in the endemic area of Peru.

Culicidae

The culicids are divided into at least two subfamilies of which the most important is that of the Culicinae or true mosquitoes. The other subfamily is that of the Corethrinae which differ from the Culicinae in not possessing a long proboscis that is adapted for piercing.

Culicinae. **Anatomical Considerations.**—Mosquitoes have three main parts of the body—the head, the thorax and the abdomen.

The head.—The space on the head behind the two compound eyes is described as consisting of two parts,—that in front being called the frons, and that behind, the occiput.

The nape is back of the occiput. The bulbous prolongation of the frons which projects over the attachment of the proboscis is the clypeus. The proboscis is straight in all mosquitoes of importance medically. In the male the puncturing parts are not sufficiently resistant to penetrate the skin. The male mosquitoes do not feed on blood but on fruits and flowers instead. The proboscis consists of a fleshy, scaled, gutter-shaped portion beneath, known as the labium, which terminates in two hinge-joint processes—the labella. At the end of the labium is a thin membrane (Dutton's membrane). It is through this that filarial embryos are supposed to pass on their way from the interior of the labium to enter the person bitten. The labium may be considered as the sheath of a knife, holding and protecting the slender, blade-like penetrating organs. Lying in this groove we have, from above downward, the horse-shoe-shaped labrum-epipharynx, the under surface of which is open. This when closed by the underlying hypopharynx forms a tube through which the blood is sucked up by the mosquito. In the hypopharynx, which somewhat resembles a hypodermic needle, is a channel, the veneno-salivary duct. It is down

this channel that the malarial sporozoite passes. The proboscis is provided with a pair of maxillae and a pair of mandibles. These four structures with the hypopharynx and the labrum-epipharynx (six structures in all) constitute the piercing parts. There are two pairs of mandibles and two pairs of maxillae on either side of the hypopharynx—the mandibles above and the maxillae below. The serrations of the maxillae are coarser than those of the mandibles. The sensory organs, the palps, lie on either side of and slightly above the proboscis. These are of importance

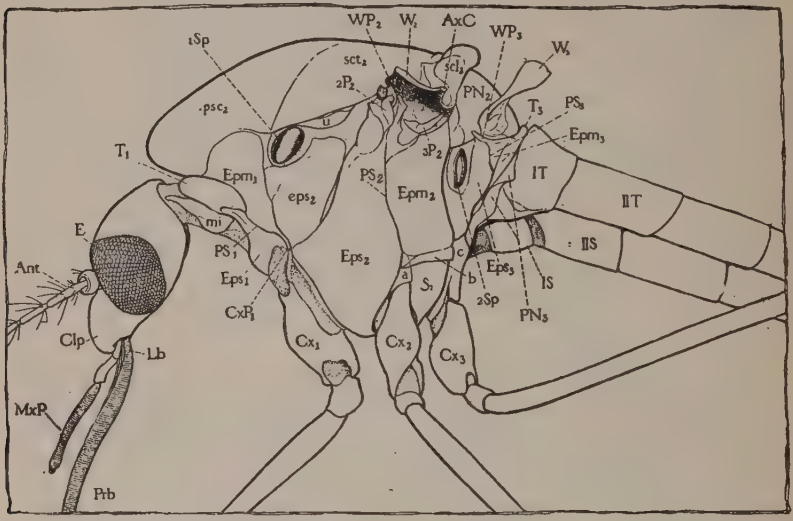


FIG. 167.—Thorax of *Psorophora*. The individual segment of the thorax to which any part belongs is indicated by the small figure placed behind and below its symbol. The abdominal segments are distinguished by Roman numerals placed before the symbols of the parts. Arabic numerals placed before symbols signify numerical order of repetition.

Ant, antenna; *AxC*, axillary cord of wing base; *Clp*, clypeus; *Cx*, coxa; *CxP*, pleural coxal process; *E*, compound eye; *Epm*, epimerum; *Eps*, episternum; *eps*$_2$, detached part of episternum of mesothorax; *Lb*, labium; *mi*, cervical sclerite; *MxPlp*, maxillary palpus; *P*, parapterum; *PN*, postnotum (postscutellum, metanotum); *Prb*, proboscis; *PS*, pleural suture; *psc*, prescutum; *S*, sternum; *scl*, scutellum; *sct*, scutum; *Sp*, spiracle; *T*, tergum; *W*$_2$, wing; *W*$_3$, halter; *WP*, pleural wing process; *a*, small plate of mesopleurum bearing articulation of coxa; *b*, accessory plate of mesoepimerum; *c*, lower part of metapleurum. (*After Howard, Dyar and Knab, by courtesy of Carnegie Institution.*)

in differentiating mosquitoes and must not be confused with the antennae, which are attached above the palpi and at the sides of the clypeus. These antennae also are of importance in distinguishing the sex of the mosquito. In the male the antennae are plumose, in the female sparsely decorated with short hairs.

The thorax.—The thorax is largely made up of the mesothorax, at the posterior margin of which is a small, sharply defined piece, the scutellum; this may be smooth or trilobed. Underneath and posterior to the scutellum is the metanotum. This

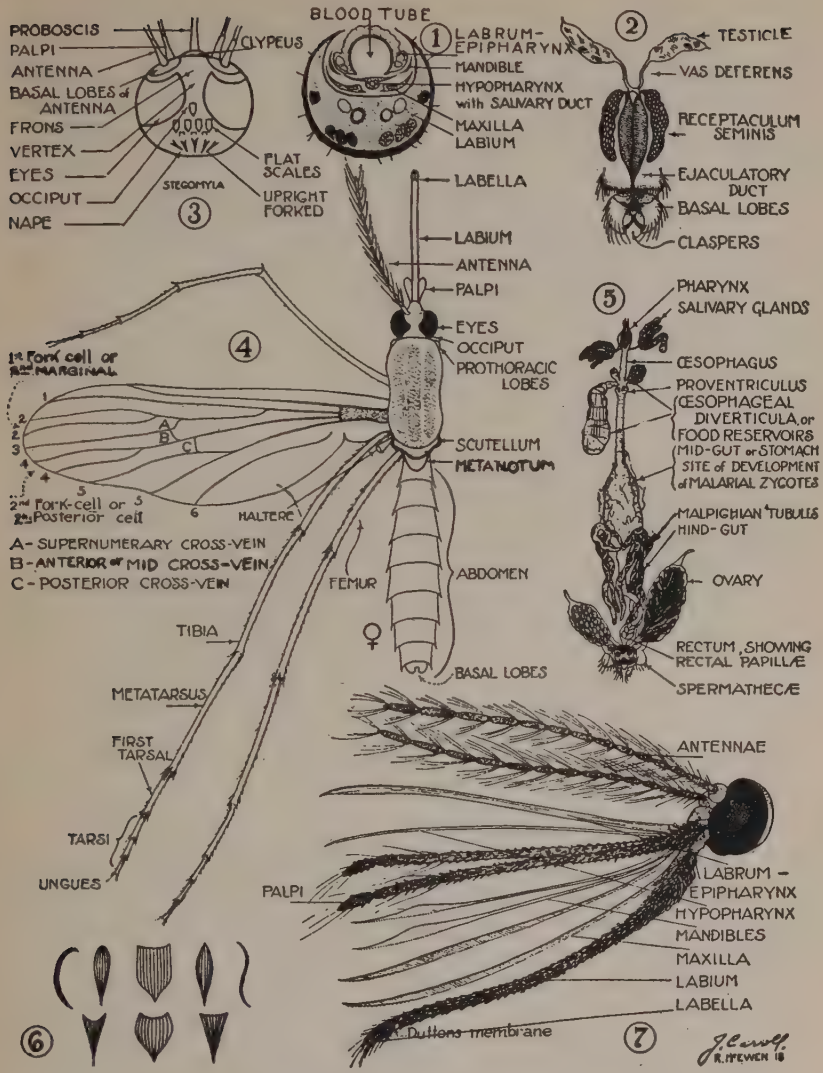


FIG. 168.—Anatomy of the mosquito. No. 6 shows various types of scales.

term implies that the structure belongs to the metathorax whereas in reality it is mesothoracic; it is often called the postnotum or the postscutellum, the latter term being usually applied in the higher orders. The metanotum is bare in the tribes Culicini and Anophelini and has a tuft of setae in the tribe Sabethini. This holds true for the species found in the United States.

There is a pair of wings attached to the posterior part of the mesothorax and, more posteriorly still, a pair of rudimentary wings (halteres) attached to the metathorax.

The *wing venation* is important. The costa shows as a stout rib or vein bordering the upper side of the wing and running around the apex and lower border.

Below, it has a fringe which may show spots. The location of the spots in the upper part of the costa of anophelines is of great value in differentiating species. Beneath the upper costal border the auxiliary or the subcostal vein runs to join the costa at some distance within the apex. The apex is the free end of the wing and the base that end attached to the thorax. Running parallel to the subcosta, but reaching the apex, is the 1st longitudinal vein. Below that is the 2d longitudinal vein

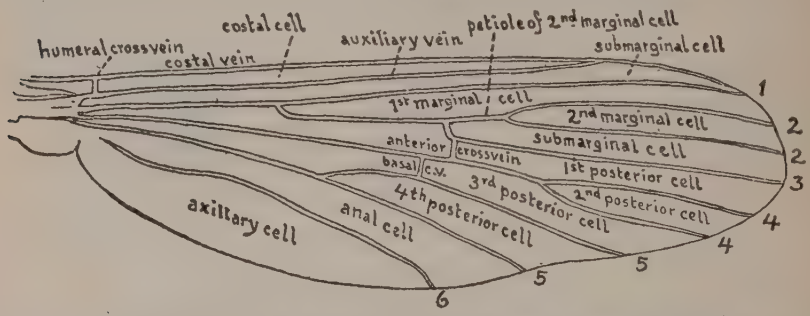


FIG. 169.—Venation of wing of *Culex*. (*From Howard, Dyar and Knab, by courtesy of Carnegie Institution.*)

which forks to make the 1st fork cell, also called 2d marginal cell. See Figs. 168 and 169. The third longitudinal vein originates from the second beyond the middle of the wing and is angulate at its base; the small transverse portion has been frequently called the "supernumerary cross vein." The 4th longitudinal divides to form the 2d fork cell (2d posterior cell). The 5th and 6th longitudinal veins arise from the base of the wing and run to the periphery. A small cross vein, which joins the basal part of the third vein with the fourth vein, is called the anterior or mid-cross vein. Another cross vein joins the fourth vein and the upper branch of the fifth vein and is called the posterior cross vein. The posterior or basal cross vein is usually a short distance behind the anterior cross vein; it may, however, be in line with it or even beyond it. The petiole or stalk of the second marginal cell is of importance in differentiating genera. The wings of all mosquitoes have scales. In addition, except in the genus *Uranotaenia*, they also possess minute hairs, microtrichia.

The three pairs of legs are attached to the thorax. Each leg has 9 parts, of which the two short ones are the basally placed coxa and the small trochanter attached to it.

Then come the long femora, tibiae and metatarsi with the four segments of the tarsi terminally. The last tarsal segment ends in two claws, which in the female may be simple or uni-serrated.

The abdomen.—There are ten segments in the abdomen. The genitalia arise from the two terminal segments as bilobed processes. The posterior abdominal appendages of the female are called the cerci, those of the male the hypopygium. In the male there is a pair of hook-like appendages or claspers, between which, and ventrally situated, are the harpes, or the 10th sternites, and also a pair of chitinous processes. See Fig. 174.

Development of the Mosquito. *The ova.*—The egg raft of *Culex*, containing about 250 ova, is quite perceptible on the surface of the water as a black, scooped-out mass, about $\frac{1}{5}$ inch in length. The eggs are set vertically in the raft. The eggs of the *Aedes* are laid singly.

Anopheles eggs are oval in shape with air-cell projections from either side. They are laid in triangle and ribbon patterns. The markings of these air cells vary and have been used for differentiation. The duration of the egg stage varies according to temperature and other conditions. The *Anopheles* are more difficult to raise than *Culex* or *Aedes*.

Larvae.—There are two great classes of larvae—the siphonate and the asiphonate. The latter are always *Anopheles*.

The *Culex* and *Aedes* larvae have a projecting breathing tube at the posterior extremity which is called a respiratory siphon. This projects off at an angle from the axis of the body, the true end of which terminates in four flap-like paddles. If you divide the length of the siphon by the breadth, you get what is known as the siphon index. The larva of *Culex quinquefasciatus* has a long and slender siphon, the larva of *Aedes aegypti* has a short and barrel-shaped one. When at the surface the *Culex quinquefasciatus* larva has its siphon almost vertical and the body at an angle of about 45°. The *Aedes aegypti* larva hangs more vertically. As a rule, the hairs proceeding from the sides of *Culex* larvae are straight and the head relatively large. There are also no palmate hairs along the sides.

The *Anopheles* larvae have a small head which is capable of being twisted around with lightning-like rapidity. They are darker in color and have no siphon; float parallel to the surface of the water; have long lateral branching hairs, and on the sides of each of the five or six middle abdominal segments they have a pair of palmate hairs. These palmate hairs are supposed to aid them in keeping their position on the surface of the water. The larvae are usually called "wrigglers." The duration of the larval stage is from one to two weeks according to the temperature.

Since larval characteristics serve as an important adjunct in differentiating tribes and genera and even species of mosquitoes, two larval keys are given under classification of mosquitoes. It is to be noted however that several of the characteristics do not appear until the last stage of larval existence, i.e., after the third moulting.

Pupae.—The pupa of the mosquito is an obtected one there being only a closely applied chitinous coating covering it; it does not have a

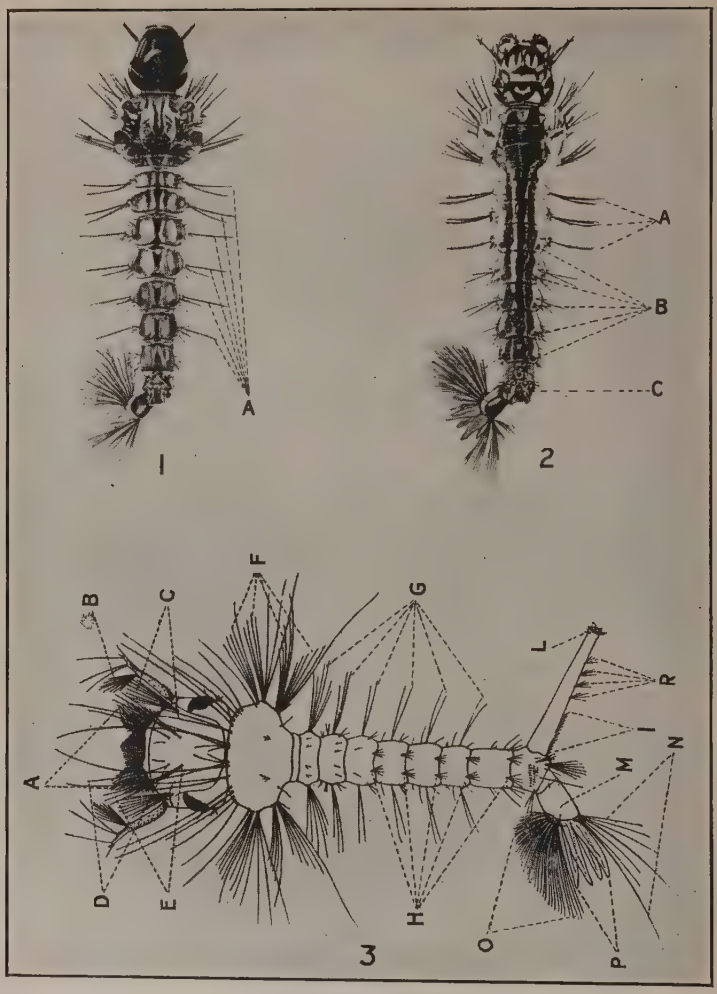


FIG. 170.—Mosquito larvae.

1. *Anopheles barberi.* (*A*) The plumose lateral hairs on the first six segments of abdomen. Note small single hairs on head.

2. *Anopheles punctipennis.* (*A*) The plumose lateral hairs on the first three segments of abdomen; (*B*) the five pairs of dorsal palmate tufts; (*C*) sessile air-tube.

3. Diagram of culicid larva. (*A*) Mouth brushes; (*B*) tuft of antenna; (*C*) ante-antennal tuft; (*D*) lower frontal tuft; (*E*) upper frontal tuft; (*F*) thoracic hair tufts; (*G*) abdominal lateral tufts; (*H*) abdominal subdorsal tufts; (*I*) pecten of air-tube; (*R*) ventral hair tufts of air-tube; (*L*) apical spine of air-tube; (*M*) anal segment; (*N*) subdorsal tufts of anal segment; (*O*) ventral brush; (*P*) anal gills. (*After Howard, Dyar and Knab, by courtesy of Carnegie Institution.*)

puparium as does the coarctate pupa of the house fly. The mosquito pupa is lighter than water while the larva is heavier.

Pupae have a bloated-looking cephalo-thorax and a shrimp-like tail—the latter being the abdomen.

The duration of pupal life is short—only one to three days. At the end of this time the pupa comes to the surface and straightens out. The integument then splits dorsally and the perfect insect emerges. It dries its wings for a time on its raft-like pupal skin and then flies away.

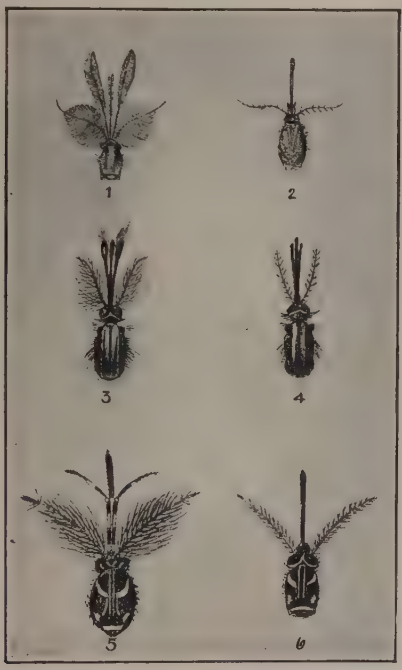


FIG. 171.—Heads of mosquitoes: 1 and 2, male and female *Culex quinquefasciatus;* 3 and 4, male and female *Anopheles;* 5 and 6, male and female *Aedes aegypti.* (*After Stitt.*) From P. H. Reports.

From the above it will be seen that the stages in the metamorphosis of the mosquito take about two weeks: One to three days for egg stage; seven to ten days for larval stage and two to three days for pupal stage.

Dissection of the Mosquito.—The easiest way to secure a mosquito for dissection is to use an ordinary test tube. Slipping the open end of the test tube over the resting mosquito, by a slight movement, the insect will fly toward the bottom. Then quickly insert a cotton plug. If it is not desired to study the scales, the best way to kill the mosquito is by striking the tube sharply against the thigh; but if it is also

desired to study the scale characteristics it is better to put a drop or so of chloroform on the lower part of the cotton plug. The vapor falls to the bottom of the tube and kills the mosquito. Take the mosquito out, pull off legs and wings, and then place the body in a drop of salt solution on a slide. It has been recommended to smear the surface of the slide with bile, wiping off the excess, before commencing the dissection in the salt solution. Then hold the anterior end of the thorax by pressure of a needle. With a second needle in the other hand, gently crush the chitinous connection between the sixth and seventh segments of the abdomen. Then holding the thorax in place, steadily and gently pull away the last segments. If this is done properly, a delicate gelatinous white mass will slowly float out in the salt solution.

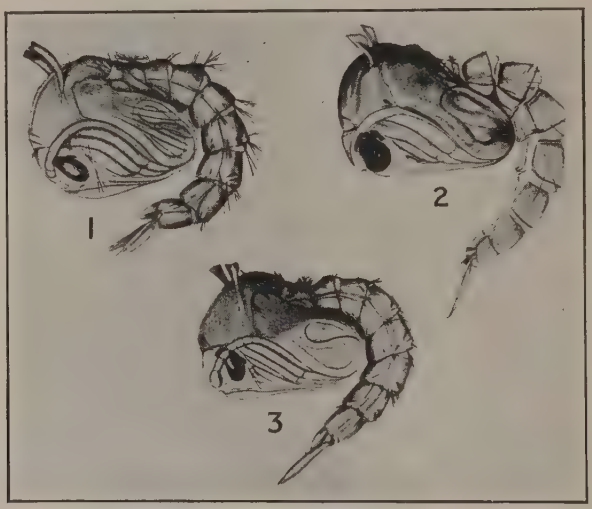


FIG. 172.—Mosquito pupae. (1) *Culex pipiens;* (2) *Aedes aegypti;* (3) *Anopheles punctipennis.* (*After Howard, Dyar, Knab, by courtesy of Carnegie Institution.*)

One should be able to secure the alimentary canal as far up as the proventriculus, which is just anterior to the stomach. The malarial zygotes develop in the stomach. Proceeding from before backward, we have the proventriculus, which is a sort of muscular ring at the opening of the stomach or mid-gut and marks the separation of the stomach from the oesophagus. Opening into the lower part of the oesophagus are the oesophageal diverticula or crops, which are food reservoirs. Occasionally in a dissection we pull out these structures which are three in number.

Leading from the stomach we have a hind-gut, which ends in the rectum.

This has a posterior dilatation or rectal pouch which usually has three or four rather marked anal papillae.

Taking origin at the posterior end of the stomach and festooning the hind-gut are five longitudinal tubes—the Malpighian tubules. These are characterized by

large granular cells with a prominent refractile nucleus. They are regarded as the renal structures. It is in these tubules that the embryo of the *Filaria immitis* of the dog develops. In the female mosquito, the parts withdrawn may seem to be largely made up of the white oval ovaries. These are connected with the spermathecae, in which the spermatozoa are stored after fecundation by the male. In the male the testicles are quite distinct. Next to the examination of the stomach for zygotes, which appear as wart-like excrescences on its outer surface, the most important structures are the salivary glands, where the malarial sporozoites are found. The easiest way to dissect out the salivary glands is to press down firmly, but gently, on the anterior part of the thorax, and then with the shaft of a second needle, pressing on the head, to gently draw the head away from the thorax, so that by this expression and traction movement you extract them with the head segment. They are very minute and are to be recognized by their exceedingly highly refractile appearance. To examine for sporozoites cover the glands protruding from the neck with a cover glass and search with one-sixth objective for narrow, curved bodies in the substance of the glands. If they are present try to smear out the glands between

FIG. 173.—Resting posture of mosquitoes: 1 and 2, *Anopheles;* 3. *Culex pipiens.* (*After Sambon.*) From P. H. Reports.

the cover glass and slide by pushing the cover glass along; then, withdrawing the cover glass, dry quickly and stain the smear on slide or cover glass with Wright's stain.

The sporozoites are narrow falciform bodies about 12μ in length, with a central chromatin dot.

A matter about which there is dispute is as to whether the salivary glands communicate with the alimentary canal. Theobald states that there is no connection between them.

Epidemiological Importance of Species Determination.—In considering whether danger might arise from the introduction into a region of a case of yellow fever, malaria or filariasis, it is essential to ascertain if genera and species capable of transmitting these diseases are present. Very definite information as to the identity of mosquitoes can be obtained, if mosquito ova are available for study, by examining specimens in the several stages of development from ovum to imago. All points concerned in species differentiation are thus made available. Having determined the species from the characteristics of egg, larva and pupa, examination of the imago becomes a process of verification.

Classification of Mosquitoes.—The classification of mosquitoes is steadily undergoing changes following progress in the science of entomology, and the discovery of new species. Theobald's classification that has been the accepted one for many years is now found wanting

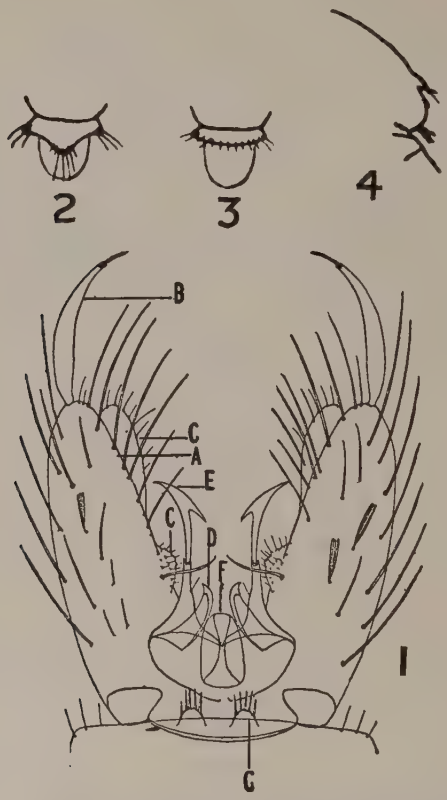


FIG. 174.—(1) Diagram of the male genitalia of *Aedes*. (*A*) side piece; (*B*) clasper; (*C*) lobes of side piece; (*D*) 10th sternite; (*E*) claspette; (*F*) mesosome; (*G*) 9th tergite. (*After Howard Dyar and Knab, by courtesy of Carnegie Institution.*) (2) *Culex* sp. The trilobed scutellum and the metanotum. (3) *Anopheles* sp. The rounded scutellum and the metanotum. (4) Sabethine species. Side view of the metanotum showing the tuft of setae.

and even misleading. The modern development in the study of larvae and their characteristics has helped much to bring order out of the chaos that existed. The scope of this book does not permit me to include the large keys necessary to identify the mosquito species of

the world. In order, however, to give the student some idea of how this identification is made, I have selected two keys for the adult mosquitoes dealing with the genera found in the United States and with species of the genus *Anopheles* found in this country; also two keys of the same scope dealing with larval characteristics.

The generic key for adults holds true only for the United States as there are some Old World Sabethines that do not conform with the classification. Even in tropical America there are Sabethines with a nude metanotum.

For the systematic study of mosquitoes of Central America, the West Indies and North America, the reader is referred to the Monograph by Howard, Dyar and Knab; and for the study of the old world species to the various publications of F. W. Edwards, of England, Theobald and others.

Key to the Tribes and Genera of the Mosquitoes of the United States. Adults. (*After Dyar*)

1. Metanotum with a tuft of setae...Tribe Sabethini Genus *Wyeomyia* Theobald
   Metanotum nude.... 2
2. Wings with the second marginal cell less than half as long as its petiole..... 3
   Wings with the second marginal cell over half as long as its petiole..... 4
3. Proboscis rigid, down curved.....Tribe Megarhinini Genus *Megarhinus* Robineau-Desvoidy
   Proboscis flexible, normal. Wings without microtrichia....Tribe Uranotaenini Genus *Uranotaenia* Lynch Arribalzaga
4. Scutellum rounded, not lobed.....Tribe Anophelini Genus *Anopheles* Meigen
   Scutellum distinctly trilobed.....Tribe Culicini 5
5. Lower side of base of first vein pilose..... Genus *Culiseta* Felt
   Without this character.... 6
6. Fourth joint of fore tarsus very short..... Genus *Orthopodomyia* Theobald
   Fourth joint of fore tarsus longer, normal.... 7
7. Second joint of antennae very long in both sexes, those of the male similar to the female..... Genus *Deinocerites* Theobald
   Second joint of antennae short; antennae of male dissimilar to those of the female.... 8
8. Abdomen of female blunt, with short cerci; hypopygium of male with the side pieces curved down, or shortly projecting.... 9
   Abdomen of female pointed, cerci exserted; hypopygium of male with the side pieces prominently projecting, straight.... 10
9. Wing scales narrow, normal..... Genus *Culex* Linnaeus
   Wing scales distinctly large and broad..... Genus *Mansonia* Blanchard
10. Abdomen of female with the eighth segment wholly retractile, nude; male claspette with multiple terminal appendages.....Genus *Psorophora* Robineau-Desvoidy
    Abdomen of female with the eighth segment only partly retractile; male claspette with only a single appendage, or claspette wanting.....Genus *Aedes* Meigen

LARVAE. (*After Howard, Dyar, and Knab*)

1. Anal segment without ventral brush, the hair tufts all paired....Tribe Sabethini
Genus *Wyeomyia*

Anal segment with unpaired ventral median brush..... 2

2. Air-tube short, sessile, the larvae surface feeders.....Tribe Anophelini
Genus *Anopheles*

Air-tube distinctly elongate..... 3

3. Head elongate elliptical.....Tribe Uranotaenini
Genus *Uranotaenia*

Head nearly circular or transverse..... 4

4. Mouth-brushes of lamellate prehensile plates.....Tribe Megarhinini
Genus *Megarhinus*

Mouth-brushes normal.....Tribe Culicini 5

5. Air-tube without pecten..... 6

Air-tube with a well developed pecten..... 7

6. Air-tube with the outer half attenuated.....Genus *Mansonia*

Air-tube cylindrical or fusiform; antennae small, slender..Genus *Orthopodomyia*

7. Air-tube with but a single pair of ventral (posterior) tufts..... 8

Air-tube with several pairs of ventral tufts, mouth brushes normal with no prehensile hooked lamellae.....Genus *Culex*

8. Air-tube pecten produced into long hairs; hair tuft close to base..Genus *Culiseta*

Air-tube pecten of short scales or if produced the hair tuft remote from base 9

9. Mandible angularly projecting laterally.....Genus *Deinocerites*

Mandible concealed..... 10

10. Anal segment ringed by plate, with ventral hair tufts piercing the ring.....
Genus *Psorophora*

Anal segment not ringed, or if so with the hair tufts posterior to the ring....
Genus *Aedes*

The only genera of importance from a medical point of view are *Anopheles*, *Aedes* and *Culex* and possibly *Psorophora*.

Anopheles.—*Determination of species.*—In addition to the generic characteristics given in the key there are several others that hold true more or less exclusively for this genus. The anopheline larvae are asiphonate surface feeders and lie parallel to the surface of the water. The imago as a rule has spotted wings. In the female the palpi are as long as the proboscis which is always straight in this genus. The body of *Anopheles* when resting on a wall forms a straight line at an angle of about 45°. It resembles a bradawl.

In contrast to the *Anopheles* the ordinary *Culex* has the following characteristics: The larvae have a siphon and the body rests at an angle of about 45° with the surface of the water. The wings of the imago are as a rule not spotted. In the female the palpi are shorter than the proboscis. In the resting position *Culex* allows the abdomen to droop, so that it is parallel to the wall. The angle formed by the abdomen with head and proboscis gives a hunchback appearance.

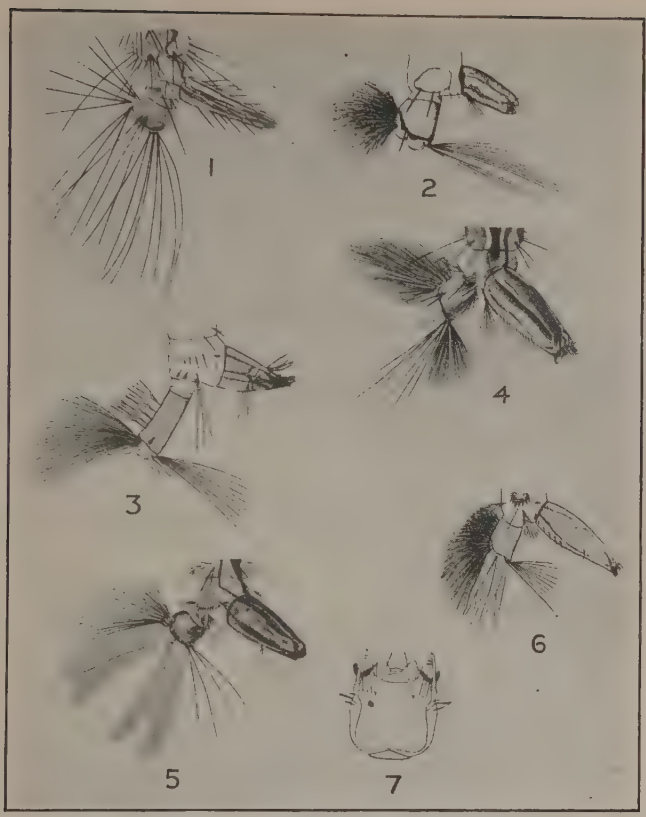


FIG. 175.—Larval characteristics demonstrating points in the keys. The posterior abdominal segments. (1) *Wyeomyia smithii*. Note absence of ventral brush from anal segment. (2) *Megarhinus portoricensis*. Note absence of pecten. (3) *Mansonia titillans*. Note attenuated outer half of air-tube, also absence of pecten. (4) *Culiseta inornatus*. Note air-tube pecten produced into long hairs; hair tuft close to base. (5) *Aedes aegypti*. Note: The ventral hair tufts on anal segment do not pierce the partial plate. (6) *Psorophora floridense*. Note anal segment ringed by plate, with ventral hair tufts piercing the ring. (7) Head of larva. *Megarhinus portoricensis*. Note the mouth-brushes consisting of lamellate prehensile plates. (*After Howard, Dyar and Knab, by courtesy of Carnegie Institution.*)

Key to the United States Species of Anopheles

Adults. (*After Dyar*)

1. Tarsi marked with white..... *albimanus* Wiedemann
   Tarsi without white markings..... 2
2. Wings with white spots..... 3
   Wings with black spots or none..... 5
3. Wings with a white spot at outer third costa..... 4
   Wings without this spot..... *crucians* Wiedemann
4. Palpi marked with white, third vein extensively white in the middle *pseudopunctipennis* Theobald
   Palpi wholly black; third vein wholly black..... *punctipennis* Say
5. Wings with black spots, usually distinct..... 6
   Wings with indistinct black spots or none, usually absent..... 7
6. Wings with a coppery spot at apex on fringe..... *maculipennis* Meigen
   Wing-fringe black at apex as elsewhere..... *quadrimaculatus* Say
7. Mesonotum rounded, but little elongate..... *barberi* Coquillett
   Mesonotum distinctly elongate..... 8
8. Palpi of female with whitish rings at bases of joints..... *walkeri* Theobald
   Without distinct white rings on palpi; body blackish; hairs of mesonotum dark-brown..... *atropos* Dyar and Knab

Larvae. (*After R. C. Shannon*)

1. Abdomen with plumose lateral tufts on first six segments; head with small simple hairs only; a tree-hole inhabiting form, New Jersey and southward.. *barberi*
   Abdomen with plumose lateral hairs on first three segments only; head with plumose hairs..... 2
2. (*a*) Abdomen with seven pairs of dorsal palmate tufts, the pair on first segment small..... *albimanus*
   (*b*) Abdomen with six pairs of dorsal palmate tufts..... *quadrimaculatus*, *walkeri*, *atropos*
   (*c*) Abdomen with five pairs of dorsal palmate tufts..... 3
3. First and last pairs of dorsal palmate tufts smaller than the others.. *crucians*
   All palmate tufts of nearly equal size..... 4
4. Elements of tufts with long slender apical portion..... *pseudopunctipennis*
   Elements not produced into long slender points..... 5
5. Lateral plate of eighth abdominal segment with 22–29 (8–9 long) teeth..... *maculipennis*
   Lateral plate with 17–22 (usually 6–7 long) teeth..... *punctipennis*

Transmitters of malaria.—The above species are all proven vectors of malaria with the exception of *barberi*, *walkeri* and *atropos*.

Anopheles albimanus.—A medium-sized black *Anopheles*, the tip of hind tarsi white with a black spot on last joint. Legs blackish, the fore tarsi with white rings at apices of the first three joints; hind legs with apex of second, third to fifth joints

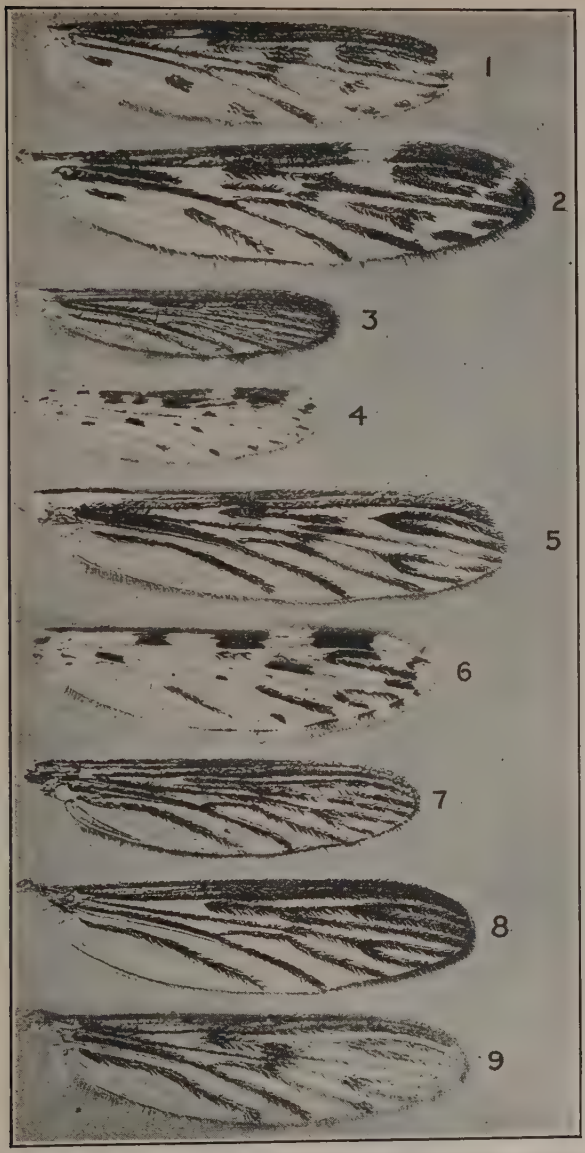


FIG. 176.—Wings of *Anopheles* mosquitoes shown to the same scale. (1) *A. crucians;* (2) *A. punctipennis;* (3) *A. barberi;* (4) *A. albimanus;* (5) *A. maculipennis;* (6) *A. pseudopunctipennis;* (7) *A. atropos;* (8) *A. walkeri;* (9) *A. quadrimaculatus.* (*After Howard, Dyar and Knab, by courtesy of Carnegie Institution.*)

white, a black mark on the fifth joint. Wings with black and yellowish scales, two large yellowish spots outwardly on costa; other veins with small dark spots alternating with pale scales. Palpi long, dark, last joint and base of penultimate one white. This is the principal vector of malaria in tropical America.

Anopheles punctipennis.—Tips of femora and tibiae with small white spots. Wings with scales black except in certain spots as follows: A large one at outer third of costa and a smaller one at apex, both involving second vein; one on third vein in the cell, on the stem and middle of both forks; at base and middle of fifth vein.

Anopheles quadrimaculatus.—A medium-sized blackish *Anopheles* with black-spotted wings. Tips of femora and tibiae whitish. Wings with the scales black, forming four dark spots by being thickly placed as follows: Base of second vein in the cell; on the cross veins and forks of second and fourth veins.

Anopheles maculipennis.—A medium-sized blackish *Anopheles* with black-spotted wings. Tips of femora and tibiae whitish. Wings with the scales black, forming four dark spots by being thickly placed as follows: Base of second vein in the cell; on the cross vein and forks of second and fourth veins. Tip of the wing with a brassy reflection in the fringe.

Anopheles crucians.—A medium-sized blackish *Anopheles* with mottled wings. Legs black with pale knee spots. Wings with a small yellowish-white spot at apex and fringe; other scales mostly black, forming spots at the bases of the forked cells and three on the sixth vein, separated by pale scales. The palpi of the female have the last joint whitish and a ring at base of penultimate joint.

Anopheles pseudopunctipennis.—A medium-sized blackish *Anopheles* with white-spotted wings. Legs black, knee spots yellowish-white. Wings spotted black and white; costa black with three white patches; third vein broadly white in the middle. Palpi of female with white rings at the bases of the joints. It greatly resembles *punctipennis*, but is not really closely allied thereto.

Another important transmitter of malaria of the New World is:

A. argyrotarsis.—It is a South American species. Black costa with two distinct and several smaller white spots. Dark-brown palps with two narrow bands and a white tip. Legs with last three hind tarsal segments white.

The following Old World species are also important transmitters of malaria:

A. funestus.—Wings with four yellow spots on a black costa and two black line spots on third longitudinal vein. Palps with three white rings. Proboscis unbanded. Legs with faint apical bands.

A. costalis.—Costa black with five or six small yellow spots. Palps with two narrow white bands and white tip. Femora and tibiae with yellow spots. Apical tarsal bands.

A. pseudopictus.—Black costa with two pale yellow spots. Wing fringe unspotted. Black palps with four pale bands. Apex of palps white.

A. fuliginosus.—Black costa with three large yellow spots. Numerous black dots on the longitudinal veins. Palpi black with white tip and two narrow white bands. Last three hind tarsal segments white.

FIG. 177.—*Anopheles maculipennis*, male. (*After Castellani and Chalmers.*) From P. H. Reports.

FIG. 178.—*Aedes aegypti*, male (*Stegomyia fasciata*). From P. H. Reports.

FIG. 179.—*Anopheles maculipennis*, female. (*Castellani and Chalmers, after Austen.*) From P. H. Reports.

FIG. 180.—*Aedes aegypti*, female (*Stegomyia fasciata*). From P. H. Reports.

Aedes and Culex.—The genera *Culex* and *Aedes* are of extreme importance medically, species of these genera transmitting filariasis, dengue and yellow fever.

Transmitters of filariasis.—The hosts for filariasis which are wholly efficient, as shown by the occurrence of filarial embryos in the thorax and proboscis, are found chiefly among the genera *Culex* and *Aedes* although anophelines also may be capable of transmitting the disease. Of the proven vectors of filariasis the following are the most important: *Culex quinquefasciatus*, *Aedes pseudoscutellaris* (Syn.: *Stegomyia variegatus*), *Aedes scutellaris* (Syn.: *Stegomyia albopictus*). Complete development of the worm has also been observed in *Anopheles rossi*, *A. ludlowi* and *A. costalis*. Flu states however that in *rossi* and *ludlowi* large numbers of the filarial larvae perish. Francis has shown that *Aedes aegypti* is an inefficient transmitter, the embryos undergoing a partial development, a fact observed in several other mos-

FIG. 181.—*Culex quinquefasciatus*, male. (*After Howard.*) From P. H. Reports.

FIG. 182.—*Culex quinquefasciatus*, female. (*After Howard.*) From P. H. Reports.

quitoes, notably many anophelines, such as *Anopheles maculipennis*, *A. bifurcatus*, *A. hyrcanus*, *A. annulipes*, *A. albimanus*, *A. argyrotarsis*.

Transmitters of dengue.—There was a general acceptance of the idea that dengue is transmitted by the common culicine mosquito of the tropics, *Culex quinquefasciatus* (Syn.: *Culex fatigans*), but in an Australian epidemic (1916) experiments failed to show *C. quinquefasciatus* capable of transmitting the disease. *Aedes aegypti*, however, gave success in 4 out of 7 cases, volunteers developing dengue in from 6 to 9 days after being bitten. Siler, Hall and Hitchens working in the Philippines definitely confirmed previous reports of the transmission of dengue by the *Aedes aegypti* and at the same time came to the conclusion that the *Culex quinquefasciatus* was not a transmitter of this disease.

The transmitter of yellow fever.—*Aedes aegypti* is the vector of yellow fever and as such is of extreme importance from a medical point of view.

Aedes aegypti (Syn.: *Stegomyia fasciata*).—The yellow fever mosquito is small in size, blackish-brown in color with silver stripes on thorax, abdomen and legs. The dorsal surface of the thorax is marked with two parallel lines with curved silver-white lines outside (lyre marking). The proboscis is black, the tips of the palpi white. There are silvery scales on clypeus.

These mosquitoes are often called domesticated, since they are observed to breed and pass their lives in the immediate environment of man and further to be distinctly urban, rather than rural, in their distribution. For their breeding places they choose artificial collections of water, such as cisterns, barrels, pails, bottles and cans, in or near dwellings.

A. aegypti is a vicious feeder and very alert. Only the female bites, blood apparently being necessary for ovulation. It feeds especially during the morning and afternoon hours—much less commonly at night unless there is a light. To become infected with yellow fever virus it must take blood from a yellow fever patient in the first two or three days of the disease. After sucking the blood of a yellow fever patient the mosquitoes cannot transmit the disease by biting a person nonimmune to yellow fever for a period of twelve days. After this time the mosquito remains infective for its life—in one instance fifty-seven days.

Aedes scutellaris (Syn.: *Stegomyia albopictus*).—This mosquito has a single silvery stripe down the center of thorax. It breeds particularly in receptacles about the house.

Aedes pseudoscutellaris (Syn.: *Stegomyia variegatus*).—This species resembles the *scutellaris* but has white bands only at the sides of the abdominal segments.

Culex quinquefasciatus (Syn.: *C. fatigans*).—A medium-sized reddish-brown mosquito. Legs and proboscis have blackish scales; the femora are pale brown at base. Abdomen blackish above, with moderate basal segmental whitish bands, separated from the lateral spots. Wing scales are narrow and hair-like, wholly dark.

The larvae occur in artificial receptacles most frequently, but are also found in ground pools, even far from habitations.

This is the common house mosquito of the tropics, extending in the Americas to about latitude 35° north and south. It is active at night.

Psorophora.—Species of this genus have been incriminated as being passive carriers of larvae of a botfly (*Dermatobia hominis*). These larvae when the mosquito alights on the skin of man emerge from the egg case, penetrate the skin and set up a cutaneous myiasis. See page 538.

Uranotaenia.—A small genus, mainly of tropical distribution. The larvae live in ground pools, and have a superficial resemblance to *Anopheles*, from the elongated black head, and the habit of lying flat in the water, although the larvae are not surface feeders. The adults are ornamented with lines of metallic blue scales.

Megarhinus.—A genus of large showy insects of tropical and subtropical distribution. The adults do not bite, the proboscis being curved and adapted to extract honey from flowers. The larvae found in tree holes and similar locations feed entirely on other mosquito larvae. The species of this genus should be classed as strictly beneficial to man. On account of their restricted habitat the species are rare.

Mosquito Eradication.—Mosquito eradication being in practice a problem of engineering and municipal administration rather than of medicine, it will suffice here to indicate the means employed, according to circumstances, in ridding a district of mosquitoes.

In rural districts, where the malaria-carrying mosquito breeds, the measure applicable are: (1) Removal of collections of stagnant water suitable for breeding by surface or subsoil drainage, by permitting free access of the tide water, or by filling in as in the case of small ponds and wells; (2) introduction of fish which prey on larvae and pupae; (3) clearing away aquatic vegetation from the banks of streams and ponds, and (4) use of physical or chemical larvicides, as oil or Paris green.

In urban and suburban districts, where the mosquitoes transmitting yellow fever, dengue and filariasis are likely to breed, the measures are: (1) Piping the water supply to remove need of cisterns; (2) screening, covering and oiling all water containers; (3) removal of all rubbish that may hold water, as bottles and tin cans; (4) drainage of surface collections of water, or, where that is not possible; (5) employment of larvicidal measures; (6) as a substitute for these measures it has been shown that the introduction of the small mosquito-destroying fish into cisterns, tanks, etc., will prevent the breeding of mosquitoes. Often fish cannot be depended upon to destroy all larvae in natural bodies of water where larvae and pupae are protected by surface vegetation.

A number of chemical agents may be employed as larvicides (See page 756). The so-called Panama larvicide formulated and used successfully by Mason is compounded as follows: Add 200 pounds powdered resin to 150 gallons crude carbolic acid. Heat mixture to 212°F. until uniform liquid is obtained. Dissolve 30 pounds caustic soda in 6 gallons of water and add to mixture, stirring briskly. Keep at boiling point until a sample immediately emulsifies with water. This larvicide in a 1 to 1,000 emulsion kills mosquito larvae in one to five minutes; in a 1 to 5,000 emulsion in 30 minutes. It should not be used with oil or to dilute oil as the soapy characteristics interfere with satisfactory filming. Efficacy is impaired by exposure to air. Costs 25 to 30 cents per gallon. It is ordinarily sprayed or sprinkled in 10% emulsion to form not less than 1 to 5,000 emulsion with the water treated.

CHAPTER XXIII

POISONOUS SNAKES

Snakes belong to the class Reptilia and the order Squamata, suborder Ophidia. The two families to which poisonous snakes belong are the colubrine snakes (Colubridae) and viperine snakes (Viperidae).

While the toxicity of the venom and the amount normally present are matters of great importance in estimating the lethal powers of species of poisonous snakes, the principal feature to be considered is the ability of the fangs to introduce venom into the tissues of the animal bitten. For example, in the Opisthoglypha there are fangs attached to the maxilla but these are placed posteriorly to the solid teeth in front so that, since the venom cannot be inoculated, these snakes are from a practical point of view nonpoisonous. Then too snakes in which the fangs are so situated have only a small poison gland and their venom is of low toxicity. In dangerous snakes the poison fangs are placed anteriorly, attached to the maxilla, which, in the poisonous Colubridae, is long and lies horizontal and, in the Viperidae, is short and lies vertical.

The nonpoisonous snakes have solid teeth only and the row of teeth is continuous; while the poisonous ones have grooved or tubular anterior teeth, the so-called fangs, behind which is a gum-margin space.

Colubrine Snakes.—Of the Colubridae the Hydrophinae or sea-snakes with rudder-like compressed tail and the Elapinae with round tails are most important.

Many of our harmless snakes such as the garter-snake and blacksnake belong to the Colubridae.

The cobras belong to the subfamily Elapinae and are best known by a neck-like expansion or hood. The only poisonous colubrine snakes in the United States are the beadsnake (*Elaps fulvius*) often called the Florida coral snake, and the Sonoran coral (*Elaps euryxanthus*).

The beadsnake is black with about seventeen broad crimson bands bordered with yellow. Although small, they are very venomous. The upper jaw has anteriorly grooved fangs, which appendages are not present in the nonpoisonous coral snakes, these latter having teeth in the upper jaw so that the wound shows four rows of punctures instead of two rows and one larger puncture on each side to mark the entrance of the fangs.

In Asia there are many important poisonous colubrine snakes, the cobra (*Naja tripudians*), the King cobra (*Naja bungarus*) and the kraits (*Bungarus fasciatus*). All of the Australian poisonous snakes are colubrines.

SNAKES OF THE UNITED STATES (STILES)

(*A*) Pupil of eye vertical; pit present; single row of ventral scales posterior to the vent; adults with head more or less triangular, constriction behind head more or less prominent.....Pit vipers (all poisonous).

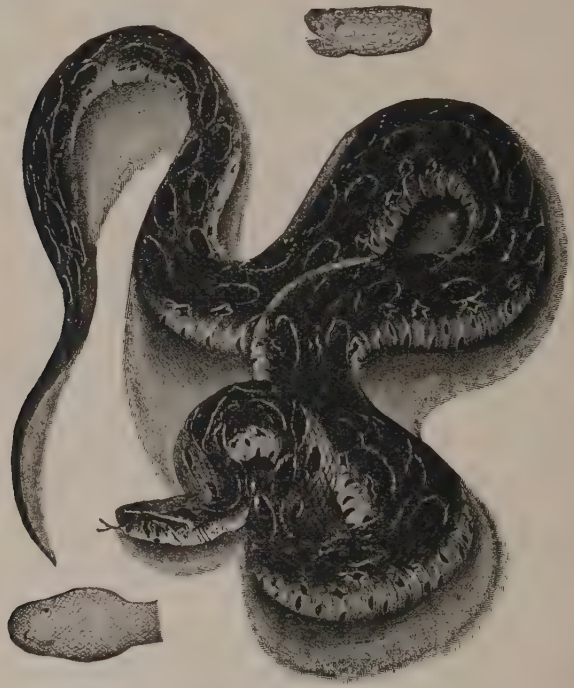


FIG. 183.—*Daboia russellii.* (*After Mense.*)

(*B*) Pupil of eye circular; pit absent; double row of ventral scales posterior to the vent.

(*a*) Color: yellow, black, yellow, red, in bands.....Coral snake (poisonous).
(*b*) Color: black, yellow, black, red, in bands....False corals (not poisonous).
(*c*) Color: all others.....Not poisonous.

Viperine Snakes.—The Viperidae which are characterized by a broad head, narrow neck, short and stumpy tail and a short upper jaw which with the fangs is directed obliquely backward. The rattlesnake (*Crotalus*), the copperhead (*Ancistrodon contortrix*), and the water moccasin (*A. piscivorus*) are widely distributed in the United States.

There are many harmless snakes which more or less resemble these "Pit Vipers," as the rattlers, moccasins, and copperheads are called. This term refers to a deep hole or pit found on the side of the head between the nostril and the eye. It is a blind sac. The much dreaded "fer-de-lance" (*Lachesis lanceolata*) is a crotaline snake.

Some divide the Viperidae into the Crotalinae, which possess the pit, and the Viperinae which do not have this structure. Russell's viper (*Daboia russellii*) is the best known of the Viperinae and is one of the most important poisonous snakes of India.

The poison fangs are grooved or perforated and connected with the poison glands which resemble salivary glands and may be almost an inch in length in large snakes. The tongue is slender and forked and is a tactile organ.

The jaws are remarkable for their great extensibility, not only vertically, but laterally, permitted by the ligamentous connections of the two halves of the mandible or lower jaw.

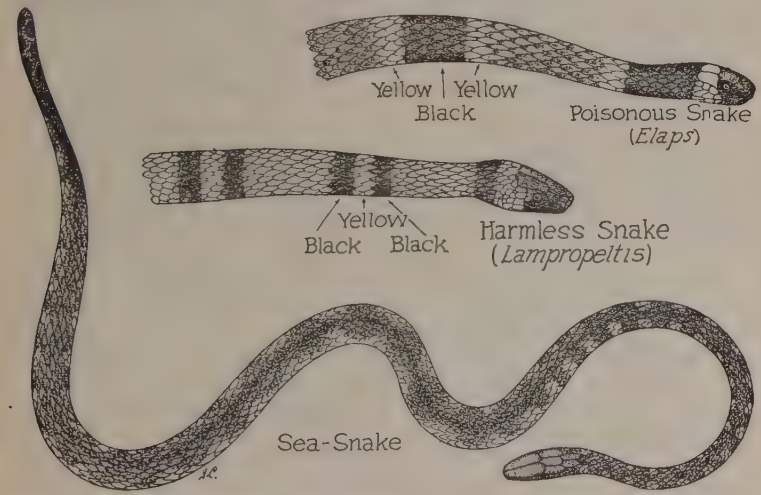


FIG. 184.—The poisonous coral snakes of the U. S., *Elaps fulvius* and *Elaps euryxanthus*, have transverse rings of black, vermillion and yellow. As differentiating these snakes from harmless ones which resemble them there are black rings bordered by two yellow ones, while with the harmless snakes a yellow ring is bordered by two black ones. The sea snake (*Enhydrina* species) has a rudder-like tail which is here shown twisted to one side.

As the fangs are directed backward it is necessary for the snake when striking to open widely the jaws and bend back the neck. The fangs are then brought forward and erected by the spheno-pterygoid muscles. The snake bite is a combination of bite and blow. The functional fangs of colubrine snakes however are not mobile.

In addition to the possession of the pit, these vipers have a more or less triangular head and in particular a single row of large scales on the under surface posterior to the vent (anus), while the harmless snakes show an elongated oval head and two rows of large ventral scales posterior to the vent.

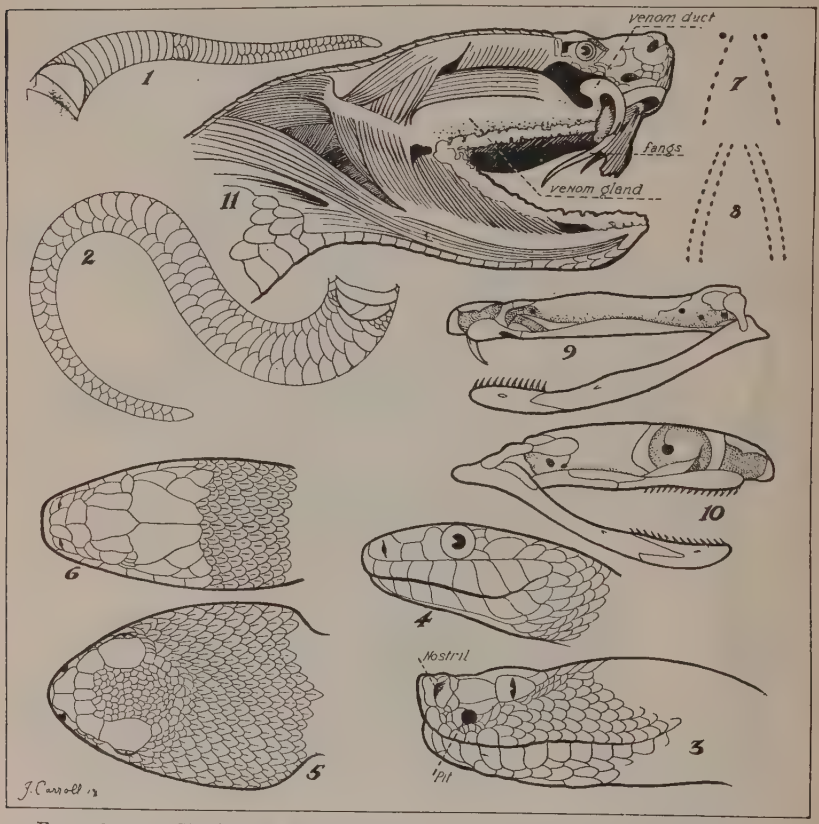


FIG. 185.—1, Single row of scales posterior to vent (poisonous snake—water moccasin); 2, double row of scales of harmless snake (*Natrix*); 3, side view head of pit viper; 4, side view head of harmless snake; 5, dorsal view pit viper; 6, dorsal view of harmless snake; 7 and 9, bite puncture and skull of *Elaps;* 8 and 10, same of harmless snake; 11, poison apparatus of rattlesnake.

Snake Venom.—In examining the wound made by a snake the two punctures of the fangs indicate the bite of a poisonous snake. If these fang-puncture points are far apart it shows that a large snake, and probably one capable of injecting a greater amount of venom, has given the bite.

When a snake strikes, the fangs move from the horizontal to the erect position, the mouth being widely open. When the fangs enter, the jaws close and pressure is exerted on the poison glands so that the venom pours out.

The amount of venom varies with the size and condition of the snake, an adult cobra yielding about 1 cc. Acton and Knowles give the following table expressed in milligrams of desiccated venom.

Common cobra (mean yield)	317.0 mgm.
Common krait (mean yield)	8.17 mgm.
Banded krait (mean yield)	64.4 mgm.
Russell's viper (mean yield)	108.0 mgm.

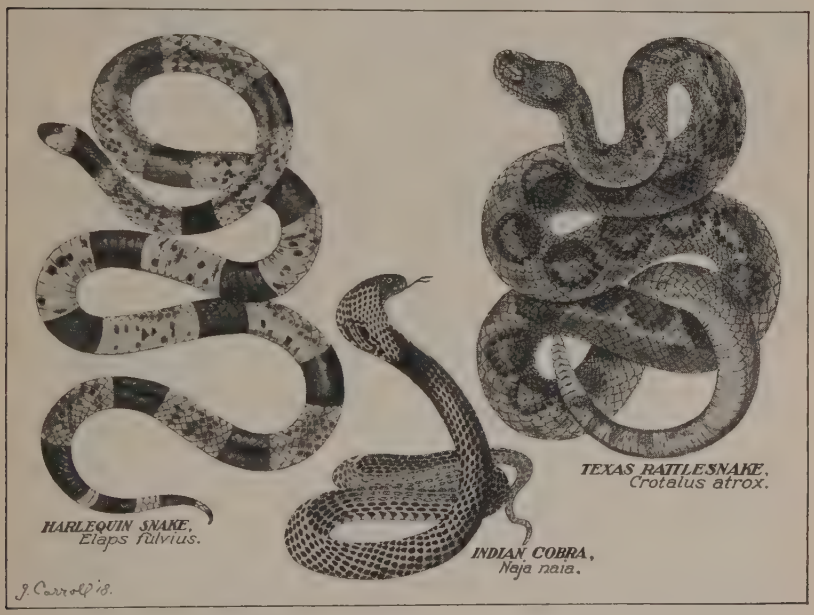


FIG. 186.—Important poisonous snakes.

They estimate the minimum lethal dose for man as 15 mgm. with cobra venom and 42 mgm. with the venom of Russel's viper (*Daboia*). The venom of the kraits is more potent, that of the very common Indian krait, *Bungarus candidus*, being given as 1 mg.

The cobra, after having bitten, remains attached for a short time while the *Daboia* strikes with the greatest rapidity and immediately releases itself.

Cobra and krait bites (colubrine snakes) produce more or less similar symptoms such as paralysis of articulation with nausea and vomiting and later paralysis of the respiratory apparatus. There is only an insignificant reaction at the point of bite.

The venom is mainly neurotoxic, causing death by paralysis of cardiac and respiratory centers. Cobra venom is also very haemolytic. This haemolysin is activated by the normal complement of the serum of the animal poisoned, the haemolysin as contained in the venom not being toxic when alone. Lecithin also has the property of activating the haemolytic amboceptor of venom.

In rattlesnake bites (viperine snakes) there is marked pain at the site of the wound with much swelling and haemorrhagic infiltration. The swelling and petechial mottling spread up the limb from the point of entrance of the venom. Cold sweats, nausea, cardiac depression, and syncope are common. An exception to this general rule is *Crotalus terrificus*, whose venom is strongly neurotoxic, affecting vision and respiratory centres. The local effects are slight.

Rattlesnake venom is active chiefly on account of its haemorrhagin, or rather endotheliolysin, which destroys the endothelial lining of blood vessels.

Venoms may also contain proteolytic ferments which may account for the softening of muscles in snake-bite cases. The toxic effect of the venom takes place without an appreciable incubation period, hence different from true toxins.

The most venomous snakes seem to be the sea-snakes (*Enhydrina*). This venom is almost entirely neurotoxic.

The tiger snake of Australia is almost equally venomous and the krait (*B. candidus*) next. The rattlesnake is about one-fifth as venomous as the krait.

Certain venoms greatly increase the coagulability of the blood so that intravascular thromboses may occur. It is chiefly with the venoms of *Daboia* and *Bungarus* that such thromboses are likely to occur and this accounts for the almost instantaneous death which at times results from bites of such snakes, when the toxin is injected directly into a vein.

Treatment.—The nonspecific treatment of snake-bite poisoning is: (1) Apply a tight ligature above the site of the bite for 20–30 minutes. The ligature, which should preferably be a rubber band, is to be applied about a single-bone extremity, not about one with two supporting bones. (2) Make deep incisions about the fang punctures and thoroughly irrigate with a strong solution of potassium permanganate. Rogers has recommended that the punctures be enlarged with a lancet and the resulting wound packed with crystals of permanganate.

Bannermann has shown that a dog bitten by a cobra cannot be saved by free incision and the rubbing in of permanganate crystals. It may however be saved by the immediate injection of 10 cc. of a 5% solution of permanganate, but not if two minutes has elapsed. Bites from the *Daboia* are fatal, however the permanganate be applied. He therefore does not consider the permanganate treatment of any practical value. Rogers thinks that Bannermann's experiments with dogs do not give a true idea of the value of permanganate because he has had success in experimenting with cats and because it has saved human lives. Chromic acid injections (1%) have also been recommended. Acton and Knowles consider potassium permanganate as unreliable and recommend subcutaneous injections of a 5% solution of gold chloride. These local injections are efficacious if used before the venom has been absorbed

but it must be understood that they have no effect on venom taken up by the circulation. Intravenous injection of permanganate is not only without effect but is dangerous. Amaral states that the ligature will not prevent the venom from spreading and may accentuate the proteolytic and cytolytic action. In his opinion permanganate solutions in active concentrations have a deleterious action on tissues.

Internally alcohol does not seem to be of any value, in fact many of the deaths have been attributed to excessive ingestion of whiskey. Strychnine in large, almost poisonous, doses was highly recommended in Australia but the statistics seem to make the value of this remedy doubtful.

Antivenins.—The active agents of snake venoms may be either of the nature of haemorrhagins, neurotoxins, or fibrin ferments. In colubrine snakes the neurotoxin vastly predominates while with the viperines it is the haemorrhagin. Certain Australian snakes contain all three bodies in about equal proportion while with the rattlesnakes of America it is almost entirely the haemorrhagin which causes the poisoning. The *Elaps* of Florida is a colubrine snake and its venom is neurotoxic in nature.

The cause of death in colubrine snake bites is chiefly from paralysis of the respiratory centers while with the pit vipers it is chiefly from haemorrhages in the vital organs. Antitoxins have been prepared against both viperine and colubrine venoms and these are specific; thus a colubrine antivenin will not be of value against a viperine bite. Antivenins should be administered either intravenously or intramuscularly. The amounts recommended for injections to neutralize a fatal dose of snake poison vary from 100 to 300 cc. of the antivenin serum. There is no accurate standardization.

Acton and Knowles recommend the following treatment for snake-bite:

(1) Apply a firm ligature immediately.

(2) Impregnate the whole area of the bite with a hypodermic injection of a strong solution of gold chloride.

(3) Inject from 100 to 200 cc. of antivenin intravenously, if the biting snake be suspected to have been a cobra or Russell's viper. If symptoms of venom intoxication come on, further and even larger injections of antivenin should be given intravenously.

With sera concentrated 10 times, a dose of 20–60 cc. should save every case of cobra bite.

CHAPTER XXIV

POISONOUS ARTHROPODS, FISH AND CNIDARIA

Venomous Arthropods

Spiders.—Spiders belong to the class Arachnoidea, order Araneida. There are numerous families, divided into various genera. As a rule spiders secrete a venom which is capable of poisoning the small animals used as food but it is only in rare instances that the venom is poisonous for man. It must be remembered that individual idiosyncrasies make one person susceptible to spider or other arthropod bites while others do not suffer.

Reports of illness following spider bites are very rare and many of these are due to secondary infections with pyogenic bacteria.

The dread of spiders is probably connected with the attributing of the hysteria of the Middle Ages, or tarantism, to the bite of *Lycosa tarantula*. As a matter of fact the bite of this spider produces only a localized erythema without general symptoms.

Experiments have shown that most of the common spiders not only are unwilling to bite but, even when almost forced to do so, are unable to penetrate other than the most delicate human skin. Even then the bite has only the effect of a pin prick.

In America we apply the term tarantula to a large, dark, hairy ferocious-looking spider of the family of Aviculariidae. The American tarantula, *Eurypelma hentzii*, is capable of killing very small animals but it is believed to be able to inflict only mechanical injury on man.

Certain species of the genus *Latrodectus* produce systemic symptoms rather than local ones. The bite of *L. Kapito*, of New Zealand, is stated to slow the pulse and respiration and produce tetanoid manifestations. In the U. S. a venomous spider, *L. mactans*, which is black, with one or more red spots on the dorsal abdomen, can produce more or less serious symptoms and possibly, but certainly most exceptionally, fatal results in man The symptoms caused by *Latrodectus* are of a nervous type although local signs at the site of the bite may also be present.

A species of *Glyptocranium*, *G. gasteracanthoides*, found in Peru, gives rise by its bite to gangrenous lesions, haematuria, etc. The bite may be fatal.

Scorpions.—These arachnids belong to the order Scorpionida. The scorpions of temperate climates are usually small but those of the

tropics may attain very large size, even 7 inches in length. The last abdominal segment terminates in a ventrally curved spine. This segment carries the poison glands.

Scorpions have formidable claws or pedipalps, with which they seize their prey and then by a downward movement of the tail-like abdomen they pierce the prey with the spine and thus introduce their venom. The poison of some of the large scorpions, as *Buthus quinquestriatus*, seems to resemble in action that of the cobra venom. While the larger scorpions are particularly to be dreaded and especially where young children have been bitten (mortality of bites of *B. quinquestriatus* in young children practically 50%), the effects of the bites of the small scorpions found in the Southern U. S. and California are probably never fatal although they may be quite painful and produce slight general symptoms.

Myriapods.—These arthropods are divided into the orders, Diplopoda, or millipedes, and Chilopoda, or centipedes. Millipedes have

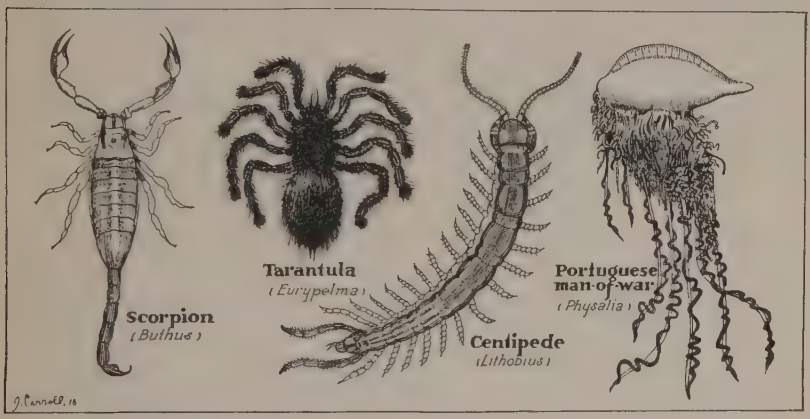


FIG. 187.—Poisonous arthropods and cnidaria.

a more cylindrical body than centipedes and, with the exception of the appendages coming from the most anterior somites, have two pairs of legs to each segment, while the centipedes have only one pair to each segment.

It is generally accepted that millipedes are harmless. Centipedes have poison glands at the base of the first pair of legs. The legs terminate in a powerful claw, at the tip of which is the opening for the expulsion of the venom. The small centipedes which are found in temperate climates rarely give rise to more than local symptoms but the large tropical ones, as for instance *Scolopendra gigantea*, which may be 10 to 12 inches long, may cause death in children by their sting and give rise to necrotic local lesions at the sites of the two punctures, and, in addition, may produce general symptoms of vomiting, headache, fever and even coma.

Bees, Wasps and Ants.—These arthropods belong to the order Hymenoptera of the class Insecta. The venom of bees is ejected through the sting which is at the end of the abdomen. In addition to the formic acid there is also a neurotoxin in the venom. When a bee stings, the biting parts are left in the wound to continue by muscular action to force out the contained venom into the tissues of the victim.

As a rule the effects of a bee sting are entirely local but cases have been reported of general symptoms ensuing, such as fever, dizziness, dyspnoea and urticarial lesions.

The bumble bee differs from the honey bee in that the sting is not cast off when stinging. Hornets and wasps have a well developed sting and are more dreaded for their sting effect than bees.

Ants.—In temperate regions ants rarely are considered as producing injury but in the tropics there are large formidable species which may not only cause local irritation but even produce general symptoms of nervous system involvement.

In the Philippines the ants are prominent factors in destroying house fly larvae so that in this way they are of great assistance to man.

Poisonous Fish

Fish Poisonous as Food.—The matter of illness produced by eating decomposed fish, whether in the natural state or canned, belongs to the general problem of food poisoning. There are, however, certain fish whose meat is poisonous when eaten in a perfectly fresh state.

This may be connected with certain epidemic diseases among fish ordinarily good food. Various bacterial organisms have been isolated from such fish and the poisonous effects have been attributed to various ptomaines elaborated by these toxicogenic organisms. Most of the organisms isolated from diseased fish have belonged to the colon or proteus groups. Cases have been reported of botulism-like poisoning arising from the eating of insufficiently salted fish. These cases were probably due to the development of a soluble toxin by *B. botulinus*, as such fish when cooked lost their toxicity. It will be remembered that the toxin of *B. botulinus* is destroyed by heat, whereas that due to the Gärtner, or ordinary food poisoning organism, withstands ordinary cooking temperatures. This fish poisoning by bacterial products is designated *ichthyotoxismus*.

The only two important animal parasite infections with which the eating of fish is connected are: (1) *Diphyllobothrium latum* and (2) *Clonorchis sinensis*. The broad Russian tape-worm is a rather common parasite of man in the Baltic provinces and comes from eating of insufficiently salted pike and other fish infected with this larval tape-worm.

The liver fluke disease of China and Japan is caused by the eating of various raw or insufficiently cooked fresh water fish. These fish are the secondary intermediate hosts, the primary ones being mollusks. A very small fluke of Japan, *Metagonimus yokogawai*, is transmitted by the ingestion of certain goldfish.

There are certain fish whose meat is poisonous when there is no question of decomposition or disease in the fish. The best known instance is with certain species

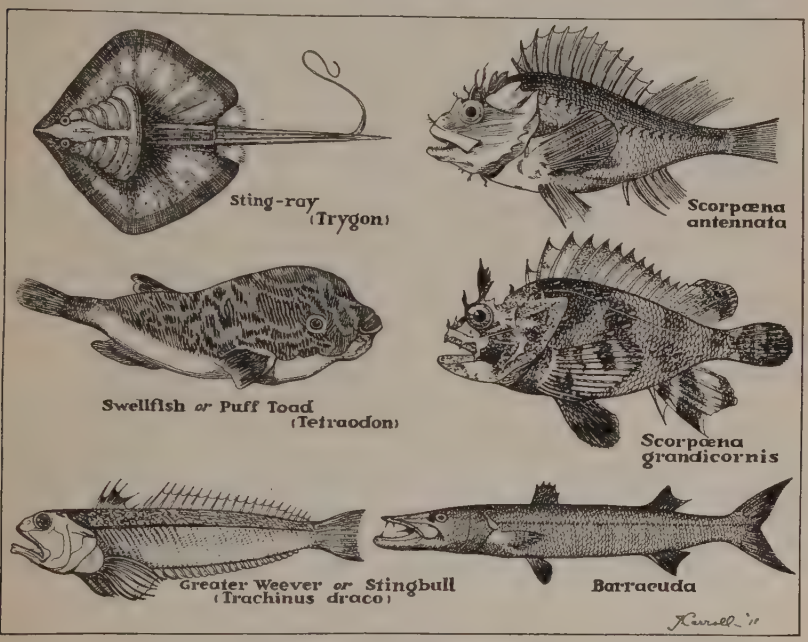


FIG. 188.—Poisonous fishes.

of the genus *Tetrodon*. The illness produced by the eating of this fish is usually termed *fuguismus*, the Japanese designating such fish by the term "fugu." The poisonous principles seem to exist chiefly in the ovaries and testicles, the eating of even one roe of such fish bringing on serious illness in a few minutes or possibly death in a few hours. It has been stated that after careful removal of all genital and alimentary tract organs these fish may be eaten without harm. The porcupine fishes or Diodontidae are considered as poisonous. These fishes together with the Tetraodontidae, or broad-nosed puffers, are unsightly in appearance. Among seamen they are generally designated puff toads as they become distended with air as they are drawn out of the water. It is well recognized that certain of these fish which may fail to cause poisoning at one time may do so at another time and it is particularly noted that poisoning effects occur at the time of spawning.

In the tropics fish which may ordinarily be safe as food become poisonous as a result of feeding on certain poisonous medusae and corals.

This is probably true of the barracuda, which is eaten with impunity at most times. Yet undoubted cases of poisoning with this fish have occurred. It has also been suggested that the barracuda may be poisonous at certain times in its life, for example, during spawning, or that it is subject to a more rapid decomposition at such times.

There are certain species of the herring family which have a bad reputation. Among these are two species of *Meletta*. In New Caledonia, *M. venenosa* causes painful cramps of the body with dyspnoea, cyanosis, cold sweats and dilated pupils and at times death. *M. theissa* of the West Indies is also a very poisonous fish.

Herre describes in the Philippine Journal of Science for October, 1924, 60 species of poisonous and worthless fishes, of the order Plectognathi.

Fish Which Poison by Their Sting or Bite.—Fish of the genus *Muraena* have well developed teeth which are in relation to a poison sac which secretes a venom which is introduced into the wound made by the bite. There are also various rays which are well known all over the world as capable of inflicting wounds.

In the sting rays (Trygonidae) the tail is armed on the upper side with a barbed spine which in some species is connected with a poison apparatus. Some of these sting rays when wounding a person who may step on them while wading in the water may at the same time inoculate tetanus bacilli which are particularly dangerous owing to the character of the deep punctured wound. In the electric rays (Torpedinidae) the dorsal surface is electrically positive and the ventral one negative. To receive a shock one must communicate with the *Torpedo* species at two distinct points. Some of these electric rays are capable of temporarily paralyzing the arm of a man.

Two of the best known poisonous fish are *Trachinus draco* and *Scorpaena scropha*. The flesh of these fish is wholesome as food. *T. draco* is like a trout in appearance and has blue and brown stripes. It has a grooved spine passing through each of its gill covers which is connected with a poison gland. There is also a poison apparatus connected with the dorsal fin. *S. scropha* is an ugly red fish with large head and prominent fins. The French fisherman call it "le diable." The poison apparatus is connected with the first three rays of the dorsal fin.

Persons in bathing who strike against these fins are more apt to be wounded than the fishermen who handle the fish with caution. Following the wounding a person experiences stabbing pains of the affected part. A sensation of suffocation follows and the victim may become delirious. At times collapse and death result. At the site of the wound we first have an erythematous area which later becomes black and may become gangrenous. As the poison rapidly enters the general

circulation treatment similar to the local treatment of a snake bite is called for. These fish seem to be more dangerous during the spawning period.

Poisonous Cnidaria

In this branch of animals the most important ones from a standpoint of medicine are the anemones and jelly fishes. A condition known as "la maladie des plongeurs" occurs among the sponge fishermen of the Mediterranean. This is due to stinging by anemones and is characterized by marked itching, burning and erythema. In some cases the skin of the affected area becomes necrotic and sloughs off leaving an ulcer.

As a rule jelly fishes are harmless but certain species produce unpleasant or even serious effects by their sting. Cases of lesions following contact with unspecified "jelly-fish" have been reported by Allen (1920) and Stewart (1922). The local rash in Allen's case was followed by profuse weeping eczema, by aphonia, and by laryngitis lasting for four weeks. Aoki (1922, 1923) portrays the severe effects, besides urticaria, of stinging by *Olindioides formosa*—shock, acute cardiac distress, dyspnoea, muscle pains, and as a possible sequela emaciation.

In the Mediterranean a jelly fish *Rhizostoma pulini* produces oedema and urticarial eruptions as the result of its sting. In many parts of the tropics jelly fishes are found which give rise to quite serious symptoms. In the Philippines there are certain species of jelly fishes which cause serious illness, although as a rule one experiences no discomfort from coming in contact with many other species while swimming in the waters of that part of the world.

According to Light, the species of *Dactylometra*, called "fosforo" by the natives, is the most dangerous one there encountered. It has long ribbon-like oral lappets and 24 slender white marginal tentacles. In this the sting is inflicted by nematocyst batteries in the four long ribbon-like oral palps. *Lobonema*, called by the natives "lanterna," is of large size, white or white and purple in color, and stings by the long filaments which arise from the mouth arms. I have treated a number of cases of jelly fish stinging in the Philippines which presented symptoms ranging from a mild erythema to those showing marked congestion of the respiratory tract and other general symptoms.

Old has described these symptoms very accurately and notes the following:

The symptoms appear in from ten to sixty minutes with marked hysterical manifestations, incessant cough and coryzal signs. Light believes that the cases described by Old were due to stinging by *Dactylometra*.

The Portuguese man-of-war (*Physalia*) has long locomotive tentacles which stretch out from 30 to 50 feet as the animal is blown along by its pearly purple crested bladder-like float or sail. The thread cells are capable of inflicting rather painful stings when handled without a knowledge of the effect of coming in contact with these thread cells.

PART IV

CLINICAL BACTERIOLOGY AND ANIMAL PARASITOLOGY OF THE VARIOUS BODY FLUIDS AND ORGANS

CHAPTER XXV

DIAGNOSIS OF INFECTIONS OF THE OCULAR REGION

It is advisable before taking material for cultures or smears to cleanse the nasal area of the eyelids, and especially about the caruncles, with sterile salt solution. Then, by gently pressing on the lids, we may be able to get pure cultures of the organism causing the infection. Normally, we may find in the region of the caruncles various skin organisms, especially staphylococci giving white colonies.

The xerosis bacillus and white staphylococci may be considered normal findings in the conjuctival sac. Streptococci and pneumococci also have been reported from apparently normal conjunctival secretions.

A small particle of sterile cotton, wound on a toothpick, with the aid of a sterile forceps, makes an excellent swab for obtaining material for smears, and may be used to make cultures on agar plates before making smears on slide or cover glass.

When there is a considerable discharge, a capillary pipette, with a rubber bulb, may be used to draw up sufficient material for cultures and smears. Be sure to round off the end of the pipette in the flame and not to use a very fine capillary tube.

Another good method of obtaining material for culture from the eye is to instil a few drops of broth and then recover it by loopfuls for inoculating media.

In conjunctival cultures, plates of glycerin agar, blood agar, or agar plates smeared with blood are to be preferred, as the *Gonococcus* and Koch-Weeks bacillus will grow only on blood or hydrocele agar. The diphtheria and xerosis bacilli grow well on glycerin agar.

In addition to the white *Staphylococcus*, the *Streptococcus* may be present when inflammation of the nasal duct exists.

The *Streptococcus* is at times responsible for a pseudomembranous conjunctivitis. The *Staphylococcus* is as a rule the cause of phlyctenular conjunctivitis.

The *Pneumococcus* is a fairly common cause of serpiginous corneal ulcerations for which active treatment is necessary.

It is now recognized as advisable to make an examination for the *Pneumococcus* before performing operations on the eye as serious results may follow if the *Pneumococcus* be present. It is the organism frequently found in dacryocystitis and, in the case of traumatism, may bring about panophthalmitis.

Corneal ulcerations are not apt to appear even with a pneumococcal conjunctivitis unless there be an injury of the epithelium.

The *B. xerosis* is possibly a harmless organism and must not be accepted as explaining an infection unless other factors have been eliminated. The true diphtheria bacillus, which the xerosis so much resembles, may cause a pseudomembranous inflammation.

The *B. pyocyaneus* may cause severe purulent keratitis as well as conjunctivitis. The pyocyaneus toxin appears to be a factor in the production of the lesions observed.

The *Gonococcus* and the Koch-Weeks bacillus are usually responsible for the very acute cases of conjunctivitis. Both these organisms are characteristically intracellular and are Gram-negative.

Conjunctivitis in the course of epidemic cerebrospinal meningitis and even panophthalmitis have been found to be due to the *Meningococcus*.

The diplobacillus of Morax and Axenfeld is more common in chronic, rather dry affections of the conjunctiva, chiefly involving the internal angle and showing a morning accumulation of the secretion. The bacilli are found in twos, more rarely in short chains. They are generally free but may be found in phagocytic cells. They resemble Friedländer's bacillus morphologically but do not have capsules.

In cases of ozaena with involvement of the nasal ducts Friedländer's bacillus may be found.

Even in cases without ozaena, capsulated, Gram-negative bacilli of the Friedländer group have been frequently reported in conjunctival inflammation and in dacryocystitis as well.

The nodules of the eyebrows give the most convenient area from which to take material in the diagnosis of leprosy, either the fluid expressed after scraping or a piece of tissue cut into sections. Conjunctival ulceration in leprosy may show abundant bacilli as is also true of corneal ulceration.

Ordinarily it is impossible to find tubercle bacilli in tuberculous conjunctival discharges.

The discharge from a tuberculous dacryocystitis may show them satisfactorily. Animal inoculation is preferable in the diagnosis of ocular T. B. The *Pneumococcus* is, however, the most important organism in dacryocystitis—rarely the *B. coli*.

In a gonorrhoeal ophthalmia the secretion is much more abundant and there is an absence of contaminating organisms, the reverse of infection with the confusing *M*

catarrhalis. As a matter of fact, large numbers of *M. catarrhalis* may be present in the conjunctival secretion with only slight irritation being observable.

B. tularense.—Wherry has reported two cases of ulcerative conjunctivitis with lymphadenitis of cervical glands, fever and marked prostration, due to infection with this organism.

In keratomycosis the cause has been ascribed to *Aspergillus fumigatus.*

Certain fungi of the genus *Microsporum* have been thought to be the cause of trachoma, as have also certain bacillary forms. One should be very conservative about reporting fungi in smears or cultures of external surfaces.

Animal parasites.—The larval form of *Taenia solium* (*Cysticercus cellulosae*) has a predilection for eye as well as brain. It is usually situated beneath the retina.

The question as to the nature of the so-called ophthalmic flukes is taken up under trematodes. *Echinococcus* cysts have been reported in the orbit.

The adult *Loa loa* tends at times to appear under the conjunctiva or in the subcutaneous tissue of the eyelids.

Fly larvae have been reported from the conjunctival sacs in the helpless sick, species of larval sarcophagids having been reported as invading the conjunctival region in purulent ophthalmias.

Demodex may cause an obstinate blepharitis.

Prowazek has thought that certain fine dots within the cytoplasm of epithelial cells, which stain best by Giemsa's method and which he considered protozoa in nature, were the cause of trachoma. See Koch-Weeks bacillus and trachoma bodies.

For *xerophthalmia* and *night blindness*, see p. 787.

CHAPTER XXVI

DIAGNOSIS OF INFECTIONS OF THE NASAL AND AURAL CAVITIES

In taking material from the nasal cavities, for bacteriological examination, it is well to wash about the alae with sterile water, then have the patient blow his nose on a piece of sterile gauze and take the material for culture or smear from this. If the material is purulent and located at some ulcerating spot, it is best to use a speculum, and either touch the spot with a sterile swab or use a capillary bulb pipette with a slight bend at the end.

Normally, we find only white staphylococcus colonies and colonies of short-chain streptococci. The *M. tetragenus*, *B. xerosis*, and Hoffmann's bacillus are also occasionally found.

In some cases of ozaena we may find an organism of the Friedländer type in pure culture.

Biscuit-shaped diplococci, both Gram-negative and positive, are to be found either normally or in cases of coryza. *M. catarrhalis* has probably been frequently reported as the *Meningococcus*. Still, the *Meningococcus*, originating most probably in the posterior pharynx, has been found in the nasal secretion of patients with cerebrospinal meningitis. *B. influenzae* (Pfeiffer) and the *Pneumococcus* have also been frequently found in cultures from the nasal secretions. The cause of the contagious type of coryza is a filterable virus.

Diphtheria involving the nasal cavity must always be kept in mind, and in quarantine investigations the examinations of the nasal secretions culturally should be a part of the routine.

The tubercle bacillus may be found in nasal ulcerations; it is, however, present only in exceedingly small numbers. On the other hand, one of the best diagnostic procedures in leprosy is to examine repeatedly smears from nasal mucous membranes for the *B. leprae*. In such ulcerations the bacilli are found in the greatest profusion. Rarely glanders may cause ulcerations.

B. proteus is frequently responsible for the production of foul odors in nasal discharges but does not seem to produce inflammatory conditions of the nasal mucosa. It simply decomposes the discharges. Various fungi have been reported from the nose, but in such a region the strictest conservatism in reporting should be observed.

A sporozoa or fungus-like organism has been reported in a case of nasal polyp. (*Rhinosporidium.*)

So many degenerative changes in epithelial cells resemble protozoal forms that such findings require ample confirmation.

The larval form of *Linguatula serrata* is a rare parasite of the nasal cavities; it is not infrequent, however, in the nostrils of dogs.

Various fly larvae are far more common, and the "screw-worm," the larva of the *Chrysomyia macellaria*, is common in certain parts of tropical America, and may by its burrowing effects cause fatal results.

The larvae of *Sarcophaga* have in particular been found in the nasal cavities of children. Myriapods, while of very little importance elsewhere, have been reported more than 30 times from the nasal fossae.

Otitis media.—In a study of the bacteriology of otitis media, in 277 cases, Libman and Celler found streptococci present alone in 81%, *Streptococcus mucosus* in 10% and the *Pneumococcus* in 8%; *Staphylococcus*, *B. pyocyaneus* and *B. proteus* have also been found. Mixed infections are common. A *Streptococcus* of the haemolytic group was an important organism in the mastoid infections so frequent during the world war.

Streptococci are the organisms which most often cause sinus thrombosis and brain abscess. The influenza bacillus of Pfeiffer has been reported as a cause of acute otitis media.

Nonvirulent diphtheroid bacilli are not infrequently obtained in cultures from ear discharges. Meningococci may cause otitis media.

Other organisms which have been isolated from middle-ear or mastoid discharges are *B. coli*, *M. catarrhalis*, *M. tetragenus* and Friedländer's bacillus.

B. typhosus may be found in middle-ear discharges of persons who have had an attack of typhoid fever.

The middle ear is normally free of bacteria, but in affections of the throat, as with streptococci, pneumococci, and diphtheria bacilli, these organisms may infect it by way of the Eustachian tube.

The moulds are of greater importance in affections of the external auditory canal than the bacteria. The cerumen seems to make a good culture medium so that various species of *Aspergillus*, *Mucor* etc., develop and may obstruct the canal. These infections are often introduced by the patient's finger. Various mites and fly larvae have been reported from the ear.

Aural myiases.—The "screw-worm," the larva of *Chrysomyia macellaria*, is the most common cause of aural myiasis in tropical America. The fly deposits its eggs about aural and nasal cavities of those with offensive discharges. The larvae gain entrance to the cavities of the head and develop, causing intense pain and giddiness. Larvae of *Sarcophaga*, *Calliphora* and *Anthomyia* have also been reported from the external auditory meatus. The tympanic membrane may be perforated by them.

CHAPTER XXVII

DIAGNOSIS OF INFECTIONS OF THE MOUTH AND PHARYNX

Mouth and Pharynx.—In a preparation made from material taken by a sterile swab from the region of the normal buccal and pharyngeal cavities and stained by Gram's method we are struck by the variety of organisms present. The fungi of thrush are best examined for in a preparation of membrane mounted in 10% caustic potash solution. *Monilia* may be found in sprue ulcerations about tongue or buccal mucosa. In examining for buccal amoebae mount the purulent material from dental alveoli in the patient's saliva. These buccal amoebae have no etiological relationship to pyorrhoea alveolaris. Smears from about carious teeth often show the fusiform bacillus and delicate spirillum of Vincent as well as cocci.

Gram-positive and Gram-negative staphylococci are normally present in pharyngeal smears as are also streptococci, pneumococci, leptothrix forms, and very probably yeasts and sarcinae types with many Gram-negative bacilli. If pseudo-diphtheria organisms are present, we have these showing a Gram-positive reaction. If this material is smeared on agar plates and cultured at 37°C., we are struck by the fact that the colonies on the plates may be exclusively staphylococcal and streptococcal.

Of course diphtheroids as well as diphtheria organisms grow well on ordinary agar plates. For *Meningococcus* carriers a blood agar plate is advisable.

It is very difficult, if not impossible, to distinguish a *Pneumococcus* colony from a *Streptococcus* one on a plate culture. In a Gram-stained smear, however, the presence of the *Pneumococcus* is easily determined by the lanceolate shape of the organism or by its possessing a capsule. It has been my experience that smears from about 15% of normal individuals show capsulated pneumococci.

In diphtheria examinations we rely chiefly on the cultural findings on Löffler's serum. In taking the culture it is better practice to rub the swab against the edge of the exudate rather than about the center. Where the process is streptococcal or due to the organisms associated with Vincent's angina, the immediate examination of a smear from the suspected spot or area gives greater diagnostic information. The *Streptococcus* being so abundant in cultures from normal throats, it is difficult to determine its significance in a culture; abundance of streptococci in a smear from an ulceration or bit of membrane, however, is of etiological import. Streptococcal sore throats are often very toxic and may be fatal—often milk-borne. Use blood agar plates to differentiate haemolyzing and "viridans" types of streptococci.

It would seem that carriers of haemolytic streptococci among the measles patients in service camps have been the chief factors in spreading this streptococcal infection to other measles cases as a result of the coughing and sneezing incident to the disease.

Measles with its tendency to bronchial irritation seems to make conditions ideal for the development of a streptococcal bronchopneumonia in those harboring haemolytic streptococci in their throats.

By staining with Neisser's method it is possible to make an immediate diagnosis of diphtheria from a smear from a piece of membrane in about 25% of cases. It is well, however, always to culture such material. The toluidin blue stain of Ponder is the best stain for diphtheria.

Material from the throat is ordinarily best obtained with a sterile copper-wire cotton-pledget swab, but sterilized applicators of wood with the absorbent cotton pledget are more frequently used. The platinum loop usually bends too easily. A sterile forceps may be more convenient for obtaining particles of membrane.

The culturing of material from the nasopharyngeal region of contacts as well as patient is very important in outbreaks of cerebrospinal fever. Use a bent wire applicator with sterile cotton tip and pass it to the nasopharynx avoiding the uvula. Inoculate tubes or plates of blood agar immediately and keep them warm until placed in the incubator.

Vincent's angina.—This is a term applied to certain ulcerative conditions of the mouth and throat, associated with the presence of a large fusiform bacillus and a delicate spirillum. The causative relationship of these organisms to the various conditions in which they are found has not been definitely established, it being maintained by some that they are secondary invaders of tissues already necrotized. Opposed to this view are the following observations: (1) These organisms are rarely if ever found in the healthy mouth; (2) they are very common in infective conditions of teeth, gums and tonsils; (3) one case at times gives rise to an epidemic of tonsillitis, all cases resembling each other clinically and showing the same organisms; (4) neo-arsphenamine, administered locally and intravenously, causes the rapid disappearance of the organisms; (5) the lesions subside coincidently with the disappearance of the organisms, and (6) the organisms injected into guinea pigs uniformly produce lesions of a constant character similar to those observed in man.

One of the organisms found in this association is the *B. fusiformis*. Two morphological types are observed. With Giemsa's stain, one type appears as a rather slender pale-blue rod with maroon dots at either end; the other is rather thicker and

shows uniform maroon staining. The bacillus is from 5 to 7μ in length, is Gram-negative and has attenuated ends. Although it is usually described as nonmotile, I have noted a sluggish but distinct motility when the material was mounted in saliva. It is an anaerobe, producing in cultures a fetid odor but no distinctive characteristics.

The identity of the spirochaetal organisms has not been determined, nor has it been settled whether several species may enter into a symbiotic relationship with the fusiform bacillus, or only one. Noguchi has cultivated from the mouth *S. microdentium*, *S. macrodentium* and *S. mucosum*. Fontana has reported a *Leptospira buccalis* in a case of noma. Kritchevsky and Seguin have made a study of the mouth spirochaetes and report four species, some or all of which may be identical with spirochaetes reported by other observers. They state that the *S. buccalis* is found rarely in the mouth and then independently of lesions, and is never found in experimental lesions in guinea pigs. It has never been cultivated. Three other spiro-

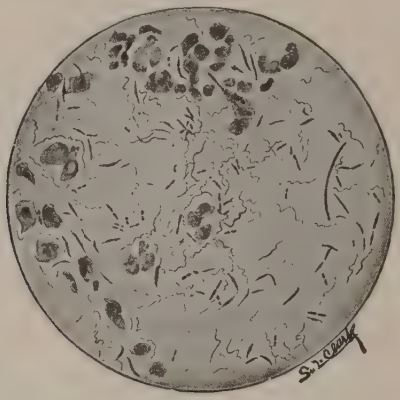


FIG. 189.—Smear from case of Vincent's angina. (*Coplin.*)

chaetes, however, the *S. dentium*, *S. acuta* and *S. tenuis*, are found commonly in lesions, are cultivable and are pathogenic for animals. The important feature of their work consists in the demonstration that an essential symbiotic relationship obtains between the fusiform bacillus and spirochaetes, and that the spirochaetal element is apparently not specific. They have showed that following the injection into a guinea pig of a pure culture of any of the three spirochaetes, together with a pure culture of *B. fusiformis*, there regularly develop lesions of a typical character consisting of local abscess formation and—often—a generalized spirochaetosis. By injecting only spirochaetes, or only fusiform bacilli, no lesions are induced. The invasion of pyogenic cocci and other bacteria was found to be a secondary phenomenon. Tunnicliff, after study of pure cultures of *B. fusiformis*, concludes that the fusiform bacilli and spirilla are different forms in the life cycle of one organism.

It appears that in man also these organisms may produce other than local lesions of the mouth and throat. They have been reported as present in ulcerative processes of the mucous membranes of the rectum and genitalia; they have been found in

ulcers of the skin; they frequently cause abscesses of the lymphatic glands, and it is quite possible that they are identical with the spirochaetal organisms found in bronchial spirochaetosis. An interesting feature of infections with these associated organisms is that they appear to provoke a lymphocytosis, the blood picture often being such that it is easily mistaken for that of acute lymphatic leukaemia.

Diagnosis is best made from smears, stained with Giemsa or Gram, by the characteristic occurrence of the two organisms in association and the absence of other known pathogens. At times, aggregations of the fusiform bacilli give the appearance of branching so characteristic of *B. diphtheriae*, differentiation then depending on the Gram stain. Pus cocci and the common mouth bacteria are always present in the smears. By mounting material obtained from the gums in saliva, motility may be observed in these organisms and also in the amoebae frequently found associated with them.

Other organisms.—Unless very familiar with the morphology of *Treponema pallidum* and using dark-field or Fontana's staining procedure, we should be very conservative in reporting such an organism from suspected syphilitic ulcerations of the throat.

We now know that we have treponemata in the buccal cavity similar to *T. pallidum* so that even with the dark-field illumination I would base a diagnosis on the clinical signs and Wassermann or Kahn reaction rather than on morphological appearances.

The thrush fungus (*Endomyces albicans*) may be easily demonstrated in a Gram-stained specimen as violet mycelial structures. Mycotic infection of the throat is not uncommon and is characterized by the presence of creamy white patches on the tonsils or pharyngeal wall. The spots are quite hard, and can be removed only with difficulty. Crushed on slides and stained they show the characteristic mycotic morphology. While not productive of much discomfort to the patient, they are very resistant to treatment.

Yeasts present in food particles are not infrequently observed in smears and cultures from the mouth.

Actinomycosis may develop about a carious tooth and the finding of the ray fungus in the granules from the pus may give the diagnosis.

Amoebae and flagellates have been reported from the mouth. For a time *Endamoeba gingivalis* (*E. buccalis*) was considered the exciting cause of pyorrhoea alveolaris, this organism being frequently obtained from scrapings about affected teeth or in the contents of root abscesses. Emetin was the accepted treatment. At present we consider the rôle of these amoebae as of no importance, and it may be added the cause of pyorrhoea is still undetermined. In the remarkable disease "halzoun," flukes have been found to be the cause of the asphyxia.

In the tropics, round worms (*Ascaris*) may be vomited up and, lodging in the pharynx, may have to be extracted.

During the campaign of Napoleon in Egypt many cases of leech involvement of the nasal and buccal cavities were noted. The parasite was the *Limnatis nilotica* which gained access to the upper pharynx through drinking water from springs and pools. Many such cases continue to be reported from the Mediterranean basin.

CHAPTER XXVIII

DIAGNOSIS OF INFECTIONS OF THE TEETH

The Teeth.—While there seems to be considerable difference of opinion, not only among medical men but also among members of the dental profession, as to the relative importance of the teeth as a source of focal infection, it is universally admitted that the teeth and their surrounding structures may and frequently do serve as an atrium; hence it is a matter of importance to observe in the conduct of examinations a procedure sufficiently thorough and systematic to enable the examiner to report with assurance that there is, or is not, infection in or about the teeth.

A method which I can recommend is as follows: Upon completion of the general physical examination, the patient is referred to the dental surgeon for an examination which consists of three main procedures:

(1) The clinical examination.
(2) The roentgenological examination.
(3) The bacteriological examination.

(1) *The clinical examination.*—There should be obtained any history of past or present subjective dental symptoms, and then there should follow a careful inspection of every tooth and its contiguous tissues by means of mouth mirrors, explorers, dental tape, and diagnostic lamps. Absence of teeth, malocclusion, caries, evidences, of periapical, lateral peridental and gingival infections, the condition of artificial restorations and the presence of calcareous deposits upon the teeth are to be noted.

An important feature of the clinical examination is the eliciting for each tooth of the reactions indicating the vitality of its pulp. These so-called vital reactions may be induced through the application of heat or cold by means of air-blasts, solutions, or solids, but it is better to employ an electric pulp-tester, which is preferable in that by control of current it is possible with it accurately to measure response. In using this instrument, however, care must be exercised in selecting the teeth to be used for controls, and in avoiding contact with materials which conduct electrical stimuli readily. While a vital pulp may be infected and the source of disease, teeth having non-vital pulps are much more likely to act as foci, and the value of the pulp-tester lies in its ability to indicate teeth which have impaired vitality and which, therefore, are most to be suspected.

Included in the clinical examination is the transillumination of the alveolo-dental structures, a step which can be carried out quickly and efficiently in the dark room.

Transillumination is valuable in that it frequently calls attention to areas of disease by changes in the amount of light transmitted.

(2) The second procedure is the taking of roentgenograms of the teeth. There is a generally held opinion that this method of examination is the most important means of determining the presence of dental disease. This may be so; but the practice of basing conclusions solely on roentgenologic appearances is not justified. For example, periapical rarefaction is an important indication of disease, yet it fails to reveal whether the changes observed are due to a disease process that is active, latent, or past.

The roentgenogram is, however, helpful in diagnosing proximal caries, in studying bony changes, in determining the form of the teeth, their positions, and their relation to other structures, in locating foreign bodies, in comparing the relative opacities of the maxillary sinuses, in observing the gross appearance of the pulp chambers and root canals of the teeth, and in orthodontic practice.

(3) *Bacteriological examination.*—In the dental clinic, or during the progress of the periodic dental examination, bacteriological methods are applied to the study of the periapical region, the lateral peridental region, and the membranous surfaces, including the gums.

Of the culture media in common use, Rosenow's glucose brain broth seems to be the most satisfactory in supplying the several conditions required to cultivate successfully the obligate and facultative anaerobes which have engaged the attention of dental bacteriologists in recent years. Correct adjustment of the hydrogen-ion concentration and oxygen tension of the culture media is essential. (See p. 36.)

In view of the difficulties met and the highly technical precautions to be observed in the management of bacterial cultures, it is believed best for the dental practitioner to depend upon a hospital laboratory or a professional bacteriologist rather than to undertake culturing himself. It is important, however, that the dental surgeon know how to obtain the material and to inoculate media.

Fortunately, much can be learned from direct examination of smears prepared by the Gram method and other simple technics. Appleton, supervising the work of students at the University of Pennsylvania, observed that the direct microscopic examination was reliable as a guide in the handling of patients requiring filling of root canals in approximately 50 per cent. of cases, although he takes occasion to point out the uncertainty of negative findings and to lay stress on the value of positive findings in contraindicating the completion of root-canal fillings.

Material from the periapical area can be obtained by aspiration through the root canal, by removal on a small instrument introduced through the canal, or after entering the area surgically through the overlying soft tissues and alveolar plate. Objections to the first and second methods are the possibility of contamination from the dentine and of killing cultures by antiseptics picked up with the material.

Among other methods of obtaining material for smears and cultures, Appleton gives the following:

(1) For culturing the periapical region.

(a) Isolate tooth with rubber dam.

(b) Sterilize coronal surface of tooth.

(c) Remove filling, temporary or otherwise, with sterile instruments.

(d) Remove filling or dressing in the root canal with sterile instruments.

(e) Mechanically cleanse the canal and finally wipe its surface with sterile paper points moistened slightly with sterile distilled water or sterile physiological sodium chloride solution.

(f) Dry the canal with sterile, dry paper points.

(g) If any moisture oozes into the canal from the periapical tissues, it may be absorbed by the application of a dry sterile paper point, which is then removed from the canal and dropped into an appropriate medium; or

If no liquid enters the canal from the periapical tissues, a sterile, fine broach or pick may be passed through the canal to pick up the material for inoculation. If a liquid medium be first inoculated, it is often well, after twenty-four hours' incubation at 37°C., to make subinoculations on a solid medium, it being often very much easier to recognize growth on a solid medium.

(2) For culturing in pyorrhoea.

(a) Isolate tooth with sterile cotton rolls, in order to minimize danger of contamination from saliva.

(b) Stroke the gingival wall of the pocket, from the apex of the tooth toward the gingival margin, to "milk" out food debris, adventitious microorganisms, and exudate. With sterile cotton pledget wipe away any material brought out.

(c) Paint the gingival margin with tincture of iodin, or brilliant green and crystal violet.

(d) With appropriate sterile instrument collect material from the depth of the pocket; or

With sterile capillary pipette collect some of the material. Dilute it in a flask containing a few cubic centimeters of sterile 1% sodium citrate solution, and transfer a few drops of this suspension with a capillary pipette to a number of deep tubes of semiliquid medium. The medium may be ascitic fluid and ordinary nutrient agar, plus preferably a small piece of fresh, sterile rabbit-kidney tissue. This method is satisfactory for the cultivation of anaerobes.

(3) Methods of culturing extracted teeth.

In obtaining cultures from infected teeth, Meisser and Gardner recommend that upon the extraction of a tooth the granuloma should be removed from the socket, immediately placed in a sterile tube and taken to the laboratory. If there is no granuloma, the tooth may be dipped in alcohol, flamed, and wrapped in sterile gauze, later to be cracked open in a vise and the culture medium then inoculated with the pulp. In pyorrhoea the material should be collected from the bottom of pockets with a capillary pipette. Material selected for culturing may be smeared on blood agar plates or inoculated into the deep glucose brain broth of Rosenow.

Infective foci about the teeth.—Of the greatest importance as constituting a menace to the general health is the presence of bacterial pathogens in apical abscesses and in the dental pulp. Pulpless teeth,

especially if the root canals have been improperly filled, are extremely susceptible to the formation of apical abscesses, and their peridental tissues are likewise liable to infection, so that the extraction of the tooth may not completely eradicate the focus of infection.

In these infections the predominating and pathogenically important organism seems to be a *Streptococcus* of the viridans-type, a green-producing *Streptococcus* appearing frequently associated with subacute endocarditis and certain types of arthritis. Unlike the haemolytic type of *Streptococcus*, which may likewise be found in such infections, the viridans type is apt to be nonpathogenic for experimental animals.

As a rule, periapical infections, whether arising from disease of the dental pulp or from the alveolodental periosteum, show the same varieties of organisms, the most commonly encountered being, as stated, the viridans group of streptococci; but diphtheroid organisms may be present, and obligate anaerobes frequently appear in cultures. The gas bacillus and the so-called *B. putrificus* have been recognized, although, owing to the difficulties of culturing anaerobes, there is little information as to their significance.

Pyorrhoea alveolaris.—This condition (known as Rigg's disease), although probably not infectious in origin, when sufficiently far advanced to exhibit recession of the gums and the formation of pus pockets is almost invariably accompanied by a characteristic secondary infection in which organisms are found in profusion and variety. In fact, this secondary infection of the soft tissues may be the earliest sign of pyorrhoea to be noticed. There is some basis for the view that these secondary invaders consist at first, or during the "dry" stage of pyorrhoea, of spirochaetes and fusiform bacilli in combination, and later of frankly pus-producing staphylococci, of pneumococci and of streptococci. The latter, when of the nonhaemolytic types, are considered by certain authorities to be the chief agents in the pyorrhoeal process.

Some believe the primary factor in pyorrhoea to be an atrophy of the alveolar margins; according to Box it is a rarefying pericementitis fibrosa; while others regard it as a manifestation of irritation due to trauma or to toxins which may be of bacterial, metabolic, or chemical nature. The significance formerly attaching to mouth amoebae is now not admitted.

There is a voluminous literature dwelling on the treatment of pyorrhoea by a vaccine made from one or more organisms isolated from the exuding pus, the streptococcal vaccines being those most commonly employed; but even when autogenous vaccines are administered the reports are most conflicting, and it is noteworthy that one point emphasized by all observers is that vaccination must always be supplemented with standard operative methods of treating pyorrhoea.

If the several procedures described are carried out whenever the question of dental infection arises, a correct opinion can be rendered as to the presence of infection about the teeth. It is, however, to be remembered, when giving advice, that the procedures outlined can have demonstrated at most only that there exists a condition such that it might constitute a focus, it being still unknown whether there is in fact a causal relationship between the dental lesion and the systemic disease. Often this question, important as it is, can be settled only after therapeutic test.

Individual resistance is an important consideration, and the dental surgeon, like the medical man, must keep in mind the patient as well as the process. Further, the physical condition of the patient must often be a great factor in determining what steps are necessary from the dental standpoint, and this physical condition best becomes manifest to the dental surgeon when he and a physician are in close professional association. Thus, such diseases as chronic infectious arthritis, pernicious anaemia, nephritis, and rheumatic fever may call for radical removal of suspected teeth with which, in less important clinical conditions, one would be justified in temporizing.

It is a well-known fact that many individuals carry demonstrable evidence of dental infection for many years without apparent interference with their health. In such cases the question of the advisability of eradicating all discoverable dental disease as a prophylactic measure is a hard one to decide. Against the advantages to be anticipated from removal of infective foci, there must be weighed the drawbacks arising from loss of masticatory agents. On the whole, considering the present state of our knowledge, it seems wise to act only on a definite indication.

Vincent's infection.—Smears of material taken from gums surrounding carious teeth, especially when the gums themselves are inflamed, frequently show the picture of Vincent's angina (see p. 585), and care should be exercised to recognize the nature of these less acute forms of this infection, and not mistake them for ordinary pyorrhoea, since the instrumentation indicated for pyorrhoea serves in this infection only to disseminate the causative organisms. In a mild case, the symptoms are those of a slight pyorrhoea dangerous only so far as the integrity of the teeth may be threatened; but it must be remembered that fulminating and exceedingly intractable gingivitis with marked constitutional symptoms may also be due to invasion of the spirillum and fusiform bacillus. The symptoms which are characteristic of the more severe forms of the infection are: (1) Pain; (2) fetor of the breath; (3) marked salivation; (4) inflamed ulcers, often covered with (5) a pseudomembrane, possibly extending to other parts, which is easily detached exposing a sensitive bleeding surface; (6) glandular enlargement; (7) fever and general malaise, and (8) a lymphocytosis. A smear should always be made in such cases. Neo-

arsphenamine, locally and intravenously, is the best preparation to control the infection.

Dental caries.—The cause of dental caries is in doubt. According to the views of Miller there is in caries a preliminary decalcification of the enamel and dentine by acids produced in carbohydrate fermentation within the mouth. The bacteria causing this production of acid were supposed to enter between the prisms of the enamel and then to traverse the tubes of the dentine. It was also thought that an enzyme produced by these bacteria was an additional factor in the development of caries. Rodriguez, basing his work on the classical researches of Miller, concludes that bacterial invasion brings about dental caries and reports as the causal agents members of the tribe Lactobacillae to which he gives tentatively the designation *Lactobacillus odontolyticus*, Types 1, 2 and 3. He considers them to be differentiated biochemically from other acid-producers of the mouth by a constant optimum H-ion concentration varying from pH 3.9 to pH 2.9. That these bacteria can effect disintegration of the mineral structures of teeth is probably due to the facts that they are active in saliva of normal reaction, produce a high degree of acidity and continue active when their environment reaches the high degree of acidity noted. On the other hand, Howe, in a discussion of the chemico-bacterial etiology, states that prolonged feeding of guinea pigs on a diet to which were added large amounts of dextrose, levulose, lactose, saccharin, dextrin and white flour showed no dental caries at the end of a year, although the sugars and starch adhered to the teeth constantly and the bacterial examination disclosed a fermentative flora. The current view of dental caries as presented recently by Howe and Grieves is that it is a process associated with faulty metabolism, especially in respect to variations from the optimal calcium-phosphorus ratio or to deficiencies in the dietary of the anti-rachitic vitamin.

CHAPTER XXIX

EXAMINATION OF SPUTUM

Frequently the material submitted for examination as sputum is simply buccal or pharyngeal secretion, or more probably secretion from the nasopharynx, which has been secured by hawking. It should always be insisted upon that the sputum be raised by a true pulmonary coughing act, and not expelled with the hacking cough so frequently associated with an elongated uvula. When there is an effort to deceive, some information may be obtained from the watery, stringy, mucoid character of the buccopharyngeal material and also from the presence of mosaic-like groups of flat epithelial cells (often packed with bacteria). The pulmonary secretion is either frothy mucus or mucopurulent material, and if the cells present are of alveolar origin they greatly resemble the plasma cells. At times these cells may contain blood pigment granules (heart-disease cells).

In the macroscopic examination of sputum we not only note color and consistence but as well the presence of such bodies as Dittrich's plugs (bronchiectasis) and Curschmann's spirals (bronchial asthma). This examination and that of an unstained wet preparation with the microscope are of value though as a rule neglected for the stained smear.

In the microscopic examination a small, cheesy particle, the size of a pin head, should be selected. This should be flattened out in a thin layer between the slide and cover glass and should be examined for elastic tissue, heart-disease cells, eggs of animal parasites, amoebae, and fungi. *Echinococcus* hooklets, Curschmann's spirals besprinkled with Charcot-Leyden crystals, and haematoidin and fatty acid crystals may also be observed.

Curschmann's spirals indicate bronchial as against cardiac or uraemic asthma. Charcot-Leyden crystals have no special significance, except in certain tropical diseases when these crystals often are present as in paragonimiasis and in the pus of amoebic liver abscesses discharging by way of the lungs.

It may facilitate the examination of the sputum for elastic tissue and actinomycosis and other fungi to add 10% sodium hydrate to the preparation. Elastic fibers are highly refractile, wavy and tend to show branching. Unless they show an alveolar arrangement we cannot be sure they do not come from elastic fibers of our food. The yellow

actinomycosis granules can be recognized by the eye and under the low power of the microscope show as nodular finely granular bodies. The threads and clubs show up with higher powers.

T. B. Staining.—To make smears for staining, the sputum should be poured on a flat surface, preferably a Petri dish, and a bit of mucopurulent material selected with forceps. A dark background facilitates picking out the particle. A toothpick is well adapted to smearing out such material on a slide. After using the toothpick it can be burned. When dry, the smear is best fixed by pouring a few drops of alcohol on the slide, allowing this to run over the surface, and then, after dashing off the excess of alcohol, to ignite that remaining on the film in the flame and allow to burn out.

A mark with a grease pencil about ½ inch from the end, gives a convenient surface to hold with the forceps and also prevents the stain subsequently used from running over the entire surface. A piece of g'ass tubing about 12 inches long bent into a narrow U-shape makes a very satisfactory rest for the slide in staining and is convenient for the steaming of staining solution over the flame.

Sputum should as a routine measure be stained by the Ziehl-Neelsen method and by Gram's method. Staining by Spengler's method permits the use of thick films.

Concentration Methods.—Tubercle bacilli usually occur nested in clumps of sputum. Therefore, when few in number it is only by chance that they may be found. Concentration methods aim to dissolve these clumps of sputum and collect, free from mucus, whatever bacilli may be present.

Mühlhaüser-Czaplewski method.—Shake up the sputum with four to eight times its volume of 0.25% solution of sodium hydrate in a stoppered bottle. When the mixture has become a smooth, mucilaginous-looking fluid, add a few drops of phenolphthalein solution and bring the pink mixture to a boil.

Then add drop by drop a 2% solution of acetic acid, stirring constantly, until the pink color is just discharged. If the least excess of acid is added over that just sufficient to cause the pink color to disappear, mucin will be precipitated. Now pour this mixture into a centrifuge tube and centrifuge; then smear the sediment on a slide and stain for tubercle bacilli.

Homogenization method.—Pottenger speaks very highly of this method. In cases, suspected of early tuberculosis, the sputum is collected for at least 24 hours. It is then homogenized by adding gasoline or xylol to 10% and shaking the mixture vigorously by means of a mechanical shaker or by hand for 10 to 15 minutes. It is now centrifuged and the sediment is examined for tubercle bacilli according to one of the accepted methods.

Antiformin method.—Uhlenluth's antiformin method has some advantages over others in the solvent used: (1) It breaks up the sputum very rapidly; (2) it immediately dissolves all organisms except acid-fast ones; (3) applied in not too concentrated form and for not too long a time, tubercle bacilli are not killed, so that by washing the sediment carefully by several dilutions and centrifugings we have in the sediment viable tubercle bacilli which we may attempt to cultivate upon Dorsett's, Petroff's, or other suitable media or inject into a guinea pig with the reasonable hope that contaminations will not choke them out or prematurely kill the inoculated

guinea pig; (4) it has less effect upon the staining properties of tubercle bacilli than any other material used in concentration methods.

To make this solvent (antiformin), proceed as in making Liquor Sodae Chlorinatae (Labarraque's solution), U. S. P., but using double the quantities of chlorinated lime and sodium carbonate called for. To the finished chlorinated soda solution add 7.5% of sodium hydrate.

The Liquor Sodae Chlorinatae of the B. P. is slightly stronger and some English authorities recommend a mixture of equal parts of this Labarraque's solution and 15% sodium hydrate solution. As a rule 1 part of antiformin to 5 parts of sputum is sufficient. Very tenacious sputum may require 1 part to 4 parts of sputum. If more antiformin is used the specific gravity is too much increased and the bacilli are damaged. The fluidification is hastened at incubator temperature.

To 5 parts of sputum add 1 part of antiformin, shake well and place in incubator for one hour. To 10 cc. of the homogeneous mixture add 1.5 cc. of a solution made up of 1 part chloroform and 9 parts alcohol. Shake violently and centrifuge for fifteen minutes. Mix the sediment with egg albumin, smear out and stain.

When it is desired to culture the tubercle bacilli mix 20 cc. of sputum with 65 cc. sterile water and add 15 cc. antiformin. Stir the mixture with a glass rod. After thirty minutes to two hours we should have a homogeneous mixture. Centrifuge for fifteen minutes or longer, wash the sediment twice with sterile salt solution and smear out the well washed sediment over serum or glycerin egg slants. The tubes should be covered with black paper and the plugs paraffined. It must be remembered that for culturing tubercle bacilli we must protect the growth from sunlight as this will kill the organism. If fluid culture media are inoculated the transferred material should be deposited on the surface. Should the particle sink, growth will not occur.

Greenfield-Anderson method.—In this 5 cc. sputum in a centrifuge tube is shaken with 10 cc. of a 1% solution of sodium carbonate in 1% phenol solution. The mixture is incubated over night and then centrifuged and the sediment stained for T. B. Raphael and Eldridge have modified this method by autoclaving the treated sputum at 15 pounds pressure for 15 minutes. Following this the material is centrifuged for 10 minutes and the sediment stained. This is claimed to show a superiority of 30% over the antiformin method. Our experience with this method has been most satisfactory.

Stained Smears.—Sputum smears stained by some Romanowsky method or by the haematoxylin-eosin stain are best adapted for the study of various cells, and in particular of the eosinophile cells so characteristic of bronchial asthma. In sputum from cancer of the lungs the large vacuolated cells may be found.

In making a smear of the tenacious rusty sputum of lobar pneumonia we note the value of a Gram stain in bringing out the capsulated pneumococci. Of course a special capsule stain may give slightly better results.

In bronchopneumonia the sputum may be somewhat streaked with blood but it rarely shows the blood admixture of the sputum of lobar pneumonia.

It is a mucopurulent sputum, smears from which are filled with pus cells, and cultures most often show streptococci as cause. In the measles bronchopneumonias which have given so high a death rate in the camps the cause is almost always a haemolytic *Streptococcus*. At times the Pfeiffer bacillus is associated with it. These bronchopneumonias have given a high percentage of empyema complications. They have also given a mortality approximating 50 per cent.

When examining the sputum of the bronchopneumonia of influenza the formol fuchsin gives the best results. The Pfeiffer bacilli are found in little masses, frequently grouped about small collections of *M. tetragenus*. The cocci stain a rich purplish-red, while the small Pfeiffer bacilli take on a light pink color.

A greenish-yellow, nummular sputum, often profuse, is frequently noted in influenza.

T. B. sputum showing a mixed infection with streptococci or pneumococci or with the Pfeiffer bacillus makes for a bad prognosis. *M. tetragenus*, which often is present when cavities exist, does not seem to be so unfavorable prognostically.

Red cells show up well in specimens stained by the Romanowsky method; if rouleaux formation is marked, it may indicate pulmonary infarction.

Culturing Sputum.—In culturing sputum a mucopurulent mass should be washed in sterile water and should then be dropped into a tube of sterile bouillon. With a sterile swab it should be emulsified and successive streaks made along the surface of an agar, blood agar or glycerin agar plate. In obtaining cultures from influenza sputum, first smear the material thoroughly over a blood-serum slant; then inoculate, by thorough smearing over the surface of successive blood-streaked agar slants, the material on the surface of the blood-serum slant. The platinum loop should be transferred from one slant to another without recharging. The Pfeiffer bacillus seems to grow better if the blood-streaked agar slants are prepared just before inoculating with the sputum. All that is necessary is to sterilize an ear, puncture and take up the exuding blood with a large loop and smear over the agar slant. Cultures for tubercle bacilli are impracticable except with the aid of antiformin or by Petroff's method. This latter is most satisfactory. A guinea pig should be inoculated.

The blood-stained watery sputum of plague pneumonia should be cultured on plates of plain agar and 3% salt agar at the same time. An ordinary smear stained with carbol thionin, however, practically makes a diagnosis. Be sure to inoculate a guinea pig cutaneously.

Pneumococci, *M. catarrhalis*, and Friedländer's bacillus in sputum are best demonstrated by Gram's method of staining.

The distinct capsule staining of the pneumococci in a Gram preparation of sputum from a suspected case of pneumonia is of value in diagnosis.

The finding of the ray fungus (*D. bovis*) in sputum gives the diagnosis of actinomycosis. *Streptothrix* infections of lungs have been confused with tuberculosis. Spirochaetal organisms may be found in great numbers in smears from bronchial spirochaetosis.

Moulds, especially aspergilli, may be found in sputum. Species of *Mucor*, *Cryptococcus*, and *Endomyces* have also been reported. In sputum from cases suspected of tuberculosis but failing to show bacilli, always examine for the organisms of blastomycosis and those of coccidioidal granuloma.

Animal Parasites.—Amoebae from liver abscess rupturing into the lung may be found. Very important pulmonary infections are those with *Paragonimus ringeri*. This is recognized by the presence of operculated eggs in the sputum.

A fluke, *F. gigantea*, was once found in sputum.

Hydatid cysts, either of the lung, or of the liver rupturing into the lung, may be recognized by the presence of *Echinococcus* hooklets. The material is bile-stained if from the liver. Dutcher has reported filarial embryos from sputum.

Metastrongylus apri has been reported once from the lungs and embryos might be found in the sputum. As the larval forms of hookworms and *Ascaris* go by way of the lungs and trachea these might be found in sputum at time of migration.

Albumin Test.—The test for albumin in the sputum is of value in the diagnosis of pulmonary tuberculosis.

About 10 cc. of fresh sputum as free as posssible from saliva is mixed with an equal quantity of water and 2 cc. of a 3% solution of acetic acid to remove mucin. After filtering, the filtrate is tested for albumin. The test is obtained also in pneumonia and pleurisy with effusion.

CHAPTER XXX

THE URINE

Material for staining is best obtained by centrifuging the urine, then pouring off the supernatant urine, then draining the mouth of the centrifuge tube against a piece of filter paper so that finally we have only to remove the pus sediment with a capillary bulb pipette, or toothpick stuck in a urine sediment pipette, and make smears.

I always take up the material with the centrifuge tube in a slanting position following the draining off of the supernatant urine to avoid urine admixture in the smear since that makes staining less satisfactory. The Gram staining is most satisfactory, counterstaining with Bismarck brown.

The addition of a loopful of egg albumin or blood serum to about twice that amount of urinary sediment gives better results. (See under Staining Methods.)

In pathological urine there is enough albumin to fix the smear.

The smear may be stained, after fixing by heat, with Gram's stain, T. B. stain, or haematoxylin and eosin. The latter is the best for the staining of epithelial cells and animal parasites; the Gram method for bacteria.

It is frequently difficult to distinguish the spores of moulds from red blood cells except by measurement and staining reactions. Spores of moulds rarely exceed five microns.

Of the greatest value is the finding of phagocytized bacteria in the pus cells of the Gram-stained smear. These indicate the causative organisms which show beautifully in the beginning of pyelitis infections. To examine for epithelial cells I make a vaseline streak across a slide about $\frac{3}{4}$ inch from the center. A drop of the sediment is deposited on the slide which may then be examined unstained with the $\frac{2}{3}$ inch objective and then a drop of Gram's iodine solution is added. One edge of a square cover glass is rubbed into the vaseline line and allowed to drop on the fluid preparation. Currents are avoided and the cells stain beautifully.

Before covering with the cover glass study the sediment with the low power for casts using low power objective and greatly diminished light. The $\frac{1}{6}$ inch objective is used after the cover glass is applied and chiefly for the recognition of small crystals, spermatozoa, red and pus cells or small epithelial ones.

It is difficult to determine the presence of blood in urine in higher dilution than 1 to 300 with the spectroscope. The ordinary occult blood test will show it in much higher dilution.

To secure urine for bacteriological examination catheterization is rarely necessary in men—in the case of women it is the proper method.

Authorities generally insist upon a catheterized specimen in all cases when the urine is to be cultured. As a matter of fact when there is a bacterial infection the specific organism is usually in such predominant numbers that it is easily distinguished from a possible contaminator. Of course should one culture the urine in a tube of bouillon, before plating out, a contamination might overgrow the causative organism, but one should always plate directly from urine which has just been passed. The smear stained by Gram's also checks up, particularly if certain bacteria are found phagocytized in pus cells. The man who follows the clinical side as well as the laboratory one is rarely confused by an occasional contaminating organism on a plate made from urine or blood. Of course the problem is more difficult with urine, but when culturing of urine is made a routine procedure the worker soon knows the organisms likely to be encountered in urine of women as well as that of men. As a matter of fact I rarely find colonies on plates made from the urine of normal men, even when the only precautions taken are those noted below. I now use blood agar as routine plating media.

The glans penis and meatus should be thoroughly washed with soap and water, after which dilute alcohol (70%) should be used. The anterior urethra may be flushed with sterile water. The greater part of the urine first passed should be rejected and only the last portion passed should be caught in a sterile receptacle. A drop of this urine may be either streaked over the surface of an agar, blood agar, or a lactose litmus agar plate, or so treated after being first diluted in a tube of sterile bouillon. The microscopical examination of the urine or the stained smear shows whether the urine should be diluted.

The lactose litmus agar medium is very useful in distinguishing typhoid or paratyphoid colonies (blue) from colon and *Streptococcus* or *Staphylococcus* colonies (pink). The urine may be added to tubes of melted agar and then poured.

The most satisfactory procedure is to deposit one drop on a poured plate and five drops on a second plate. The surface is smeared over with a bent glass rod first smearing out the single drop and then going to the second plate without a second sterilization. Neutral glycerin agar or blood agar is desirable for such organisms as pneumococci or streptococci and, for the *Gonococcus*, certain special media.

Cystitis from a colon infection gives an acid urine; that caused by *Proteus vulgaris* an alkaline urine.

The old designation *B. termo* so often employed in connection with the bacteriology of the urine in older works applied to the proteus group, and *M. ureae* to ordinary staphylococci.

Gonococci are reported from Gram-stained smears. Coccoid forms or phagocytized fragments of colon bacilli may be confused with gonococci.

To culture *Gonococcus* material the transfer to culture media should be made almost immediately after obtaining the material from the patient. *M. catarrhalis* is a rare finding.

The bacillus of typhoid and the micrococcus of undulant fever are also found in the urine. This elimination in urine of bacilli by typhoid carriers is of great importance in the spread of the disease.

Acid-fast organisms.—While the smegma bacillus in urine may be differentiated from the tubercle bacillus by the former losing its red color by prolonged decolorization with acid alcohol, yet it is chiefly by the subcutaneous inoculation of the guinea pig that we should diagnose genito-urinary tuberculosis. (See p. 130.) Inject the sediment after centrifuging. I have had smegma bacilli withstand prolonged decolorization. In a man the finding of acid-fast organisms in urine sediment, taken as noted above, almost surely means genito-urinary tuberculosis. In women catheterization of the ureters will exclude smegma bacilli.

The method recommended by Gasis which depends on the alkali-fast properties of the T. B. has not given me satisfactory results.

Smegma bacilli are not disintegrated by antiformin as are other bacilli than the tubercle one, so that treatment of urinary sediments with antiformin for finding tubercle bacilli does not differentiate those of smegma.

Ellerman and Erlandsen have devised a very reliable method for the detection of tubercle bacilli in urine.

a. Having washed genitalia and irrigated urethra, draw urine by catheter; allow to settle several hours.

b. Decant. Take 20 cc. of sediment; centrifuge and decant.

c. Sediment is mixed with four times its bulk of 0.25% solution of sodium carbonate and 0.5 Gm. digestive pancreatin; incubate 24 hours.

d. Upper layer of fluid is decanted; remainder is centrifuged.

e. Decant fluid. To sediment remaining, add four times its volume of 0.25% NaOH, and stir until sediment is dissolved.

f. Heat to boiling over a water bath for several minutes. Let cool. Centrifuge. Make smears from sediment and stain, preferably by Spengler's method.

Another good method.—Boil all ware for one hour in 25% NaOH solution, and wash with freshly distilled water until blue litmus remains unaffected.

1. Clean urethra by irrigation to remove smegma. Wash genitalia carefully with green soap and water.

2. Collect urine in 100 cc. lots, taking the first and the last hundreds. Run through separately.

3. Allow to sediment in incubator (to keep urates soluble) for twelve hours. Add a little boric acid; or, if much sediment be present, 10 cc. of antiformin.

4 Pour off fluid; take up the whole of the sediment and centrifuge at high speed for five minutes.

5. Pour off fluid. Add alcohol 10, HCl 5, distilled water 85.

6. Shake and centrifuge.

7. Pour off fluid. Add distilled water 90, alcohol 10.

8. Shake and centrifuge.

9. Pour off fluid. Take up sediment for slides.

Fix slides as usual. Stain as usual, using acid-alcohol for decolorizing, and an extra wash with alcohol after acid-alcohol.

In *Staphylococcus* and *Streptococcus* infections about the throat as well as such infections in heart or joint, the causative organisms may be present in the urine. At times bacterial infections of the kidney may give symptoms of renal stone.

It is much easier to secure urine for culture than blood, and a bacteriological examination of the urine may suffice to give us the desired information and the organism for the autogenous vaccine. Salt mouth bottles with cotton plugs, when sterilized, make cheap and satisfactory containers. The urine should be plated out as soon as possible after its passage. As a rule when organisms are present in the urine they are in such numbers that the question of contamination rarely arises.

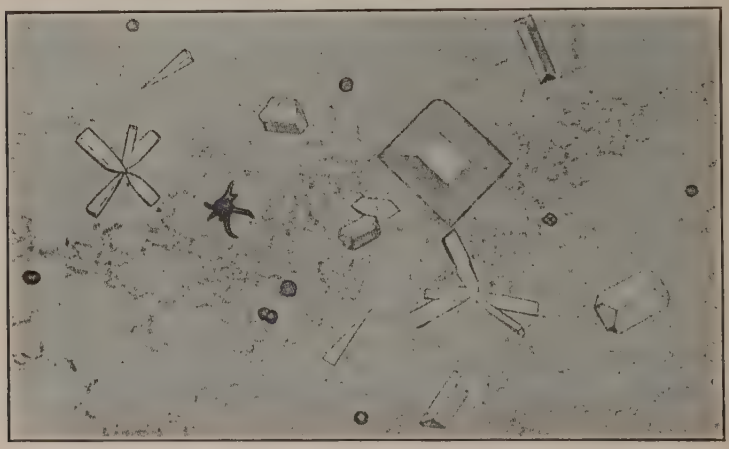


FIG. 190.—Common sediments of alkaline urine: Triple phosphate crystals. calcium phosphate crystals, ammonium urate crystals, and amorphous phosphates. ×150. (*J. C. Todd "Clinical Diagnosis."*)

Yeasts and moulds frequently contaminate urine, especially diabetic urine, after it has been passed. Amoebae and flagellates (*Trichomonas vaginalis* in females) may be found in urine.

Eggs of *Schistosoma haematobium* (bilharziasis) are important diagnostic findings; these are terminal-spined. Those of rectal bilharziasis are, as a rule, lateral-spined.

In chylous urine the filarial embryos may be found. This examination is facilitated by centrifugalization.

The eggs of the *Macracanthorhynchus hirudinaceus* may be recognized in urinary sediment by their pitted appearance.

The vinegar eel may be found in the urine of females who have used vaginal douches of vinegar.

Echinococcus hooklets, scolices, or laminated membrane have been found in the urine.

The larval dibothriocephalid, *Sparganum mansoni*, has been reported three times in urine (urethra).

Enterobius from the vagina may be found in urine.

Various mites may be found in urinary sediment as the result of lack of care in the washing of the receptacle and are entirely accidental.

Unless having the characteristics of the itch mite and in a person showing scabies lesions about the genital organs the diagnosis of the mite as *Sarcoptes scabiei* should not be made.

Crystals of biliverdin may be found in the urinary sediment in marked jaundice. They somewhat resemble crystals of tyrosin but are brownish in color while those of tyrosin are black. Furthermore, it is excessively rare to find crystals of leucin and tyrosin in the urinary sediments, and in such diseases as acute yellow atrophy of the liver, the urine should be concentrated to one-tenth its volume and the residue treated with alcohol. The tyrosin crystalline sheaves and the leucin striated globules crystallize out from the alcohol.

Urinary Sediments

Turbidity of the urine is most often due either to bacterial contamination, amorphous urates (brick-dust sediment; sedimentum lateritium) or phosphates.

In case turbidity is found, due to bacteria contaminating the urine subsequent to its passage, it is best to call for another sample.

To preserve urinary sediments formalin is the best for casts and epithelial cells while for general use one may employ a piece of camphor or add one volume of saturated borax solution to four volumes of urine.

To take up a sediment insert a pipette to the bottom of the tube with the opposite opening closed by a finger, then tease the sediment into the pipette opening by manipulating the finger; then, holding the finger on the open end tightly, withdraw the pipette and deposit the sediment on a slide.

Mounting a sediment in Gram's solution or tinging it with the merest trace of neutral red is of much assistance.

Unorganized Sediments.—These are, as a rule, of little clinical interest, and give no reliable indication of abnormal production or excretion. The precipitation of any particular substance is dependent upon many physical as well as chemical factors, of which the reaction is very important. In amphoteric urines, one may encounter elements usually associated with either an acid or an alkaline reaction.

In a urine of acid reaction, we may find the following: (1) Amorphous sodium or potassium acid-urates. Usually yellowish-red. Heat and alkali cause solution. (2) Uric acid. Yellowish-red crystals, usually of a whetstone shape and in clusters or heaps. Soluble in alkalies but not by heat. (3) Calcium oxalate. Highly refractile octahedral crystals, or in dumb-bell shapes. Often due to diet (asparagus, tomatoes, spinach, rhubarb, etc.). Clumping of the crystals is suggestive when

calculus is suspected. (4) Rare crystals, such as cholesterol, cystin, tyrosin, leucin, xanthin, haematoidin, indigo, melanin, creatinine, hippuric acid, sodium biurate, $CaSO_4.2H_2O$, neutral calcium phosphate ($CaHPO_4.2H_2O$), etc.

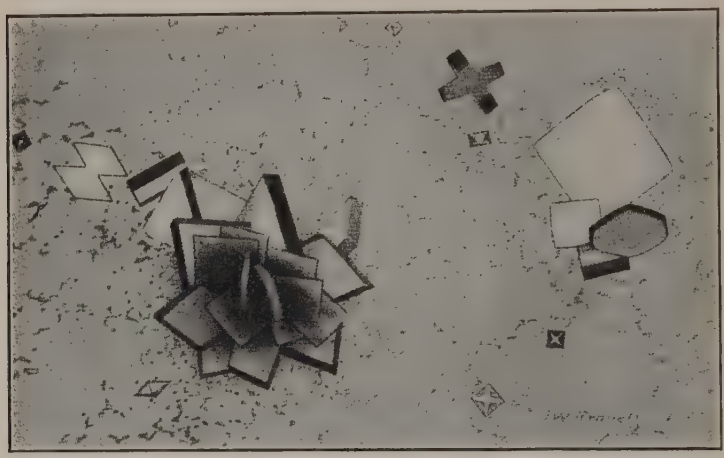


FIG. 191.—Common sediments of acid urine: Uric acid crystals, calcium oxalate crystals, and amorphous urates. ×150. (*J. C. Todd "Clinical Diagnosis."*)

In amphoteric urine, one may encounter dicalcium phosphate, or practically any other crystal.

FIG. 192.—Unusual urinary crystals (drawn from various authors): 1, Calcium sulphate (colorless); 2, cholesterol (colorless); 3, hippuric acid (colorless); 4, haematoidin (brown); 5, fatty acids (colorless); 6, indigo (blue); 7, sodium urate (yellowish). (*J. C. Todd "Clinical Diagnosis."*)

In urine of alkaline reaction, we may expect: (1) Triple phosphates ($NH_4MgPO_4.6H_2O$). Usually in coffin-lid crystals, or in fern-like forms. Easily soluble in acetic acid. (2) Tri-calcium phosphate [$Ca_3(PO_4)_2$], magnesium phosphate

$[Mg_3(PO_4)_2]$, and calcium carbonate. All are amorphous and easily soluble in acetic acid, the last with effervescence. (3) Ammonium biurate. Yellow, thorn-apple structures. (4) Calcium phosphate. Slender, radiating crystals, or flat sheets. (5) Rarely, magnesium phosphate crystals.

The presence of ammonium biurate, particularly if with triple phosphates, denotes bacterial decomposition within the genito-urinary tract provided the urine is freshly passed. Pus cells derived from the site of inflammation should be present also. While certain bacteria might possibly cause chemical changes without giving rise to inflammation, yet such a possibility is so rare as to be negligible. If amorphous phosphates are found, one should always consider exogenous sources such as vegetable diet, or special causes, as withdrawal of protein food, before diagnosing disordered metabolism.

Leukocytes.—An occasional leukocyte may be found in the urine of healthy people. An abundance of leukocytes indicates inflammation of genito-urinary tract. Some workers count the pus cells in urine

FIG. 193.—Epithelium from different areas of the urinary tract. *a*, Leukocyte (for comparison); *b*, renal cells; *c*, superficial pelvic cells; *d*, deep pelvic cells; *e*, cells from calices; *f*, cells from ureter; *g*, *g*, *g*, *g*, *g*, squamous epithelium from the bladder; *h*, *h*, neck-of-bladder cells; *i*, epithelium from prostatic urethra; *k*, urethral cells; *l*, *l*, scaly epithelium; *m*, *m'*, cells from seminal passages; *n*, compound-granule cells; *o*, fatty renal cell. (*Ogden.*)

by the same technique used for the leukocyte count of the blood. At times, the urine of women may contain an abundance without pathological significance.

Erythrocytes.—These may retain their biconcave form, be crenated, or only show as pale, possibly double-ringed bodies, termed "shadows."

They are, however, usually quite uniform in size, a fact that will aid when one encounters, as he frequently does, other structures that closely resemble them, such as atypical calcium oxalate crystals. Vegetable spores are often a source of confusion. It is always well to check a positive finding by a test for occult blood.

The menstrual period must be kept in mind. Red blood cells also appear in inflammations, particularly stone or schistosome infection; also with neoplasms, or in chronic passive congestion, as well as in conditions in which toxins are being eliminated, such as tuberculosis.

Epithelial Cells.—For morphology of cells from different locations, see Fig. 193. It is almost impossible to state positively the origin

FIG. 194.—Fatty and waxy casts. *a*, Fatty casts; *b*, waxy casts. (*Greene.*)

in the genito-urinary tract of certain cells. Very trustworthy evidence, however, is the finding of epithelial cells on casts or the so-called compound-granule cells (fatty, degenerated renal epithelium). Sheets of more or less round or caudate epithelial cells are rather significant of pyelitis. Vaginal epithelium resembles that secured by scraping the buccal mucosa. Bladder epithelium resembles vaginal but is of smaller size; ureteral is like that from the pelvis of the kidney, but

smaller. Cells from the region of the prostate are very refractile, have a distinct nucleus, and are oval rather than round.

Cylindruria.—This means the presence of cylindroids or casts. It will be found that a $\frac{2}{3}$ in. objective gives almost all the information required. See albumin (p. 726) for further consideration of its significance. Cylindroids are long structures resembling hyaline casts but showing tapering ends, irregularity of diameter, and longitudinal striations. They have the same significance as hyaline casts. Casts are cylindrical structures (Fig. 194) with rounded ends. One must bear in mind their fragility. It is said that prolonged centrifuging will disorganize them, and it is certain that they disappear during ammoniacal fermentation. They are of a light specific gravity and tend to occur in the upper portion of the sediment, either in the centrifuge tube or on the slide. Todd says, "If the tubule be small and has its usual lining of epithelium, the cast will be narrow; if it be large or entirely denuded of epithelium, the cast will be broad." Persistent presence is of graver significance than occasional occurrence.

Hyaline casts are narrow and homogeneous. They follow almost any renal disturbance, and are not necessarily indicative of any permanent damage. Finely granular casts may have no greater significance than hyaline, and, as a matter of fact, the latter show a granular structure with dark-ground illumination. As the granules become coarser, it is generally considered that a more severe lesion is present, especially of a chronic, parenchymatous type. Fatty casts are especially associated with fatty degeneration. Epithelial casts usually indicate a parenchymatous inflammation, their number being an index of its severity. Blood casts and pus casts mean a serious kidney lesion. Waxy casts are highly refractile, and show fissuring of the margins. They may accompany a large white kidney or a severe acute nephritis, but, on the other hand, they may simply be ordinary casts that have been retained in the tubules for a long time. Even amyloid kidney does not produce any distinctive cast.

Starches and Fibers.—In examining urinary sediments it is important to be familiar with the various textile fibers and starch grains which are so frequently present, the fibers coming from the clothing and the starch grains from dusting powders. Wool fiber fragments show bark or scale-like imbrications and are round. Cotton fibers are flattened and twisted, while linen ones show a striated flattened fiber with frayed segments as of a cane stalk. Silk shows a glass-like tube with mashed-in ends.

Corn and rice grains are the most common of the starch grains and their nature is immediately disclosed by their blue color when mounted in iodine.

Haematuria.—By this we mean the presence of red blood cells in the urine, a condition often recognizable only by microscopic or occult blood examinations. It is essential to discriminate between this condi-

tion and haemoglobinuria. In origin blood may be renal, cystic, urethral, ureteral and secondary to disease or traumatism. It may also result from affections of structures adjacent to the genito-urinary apparatus, especially ulcerations of the large intestines (amoebic), or from disease of parts of the female generative apparatus as uterus, vagina or tubes.

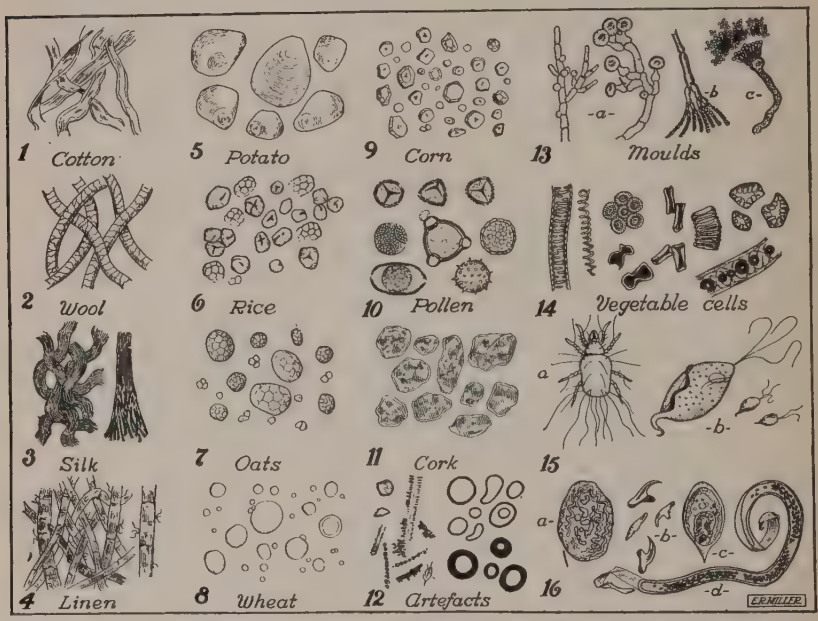


FIG. 195.—Fibers, starch granules, etc., which may be found in urine sediment. No. 12 gives appearance under microscope of scratches on old used glass slides. No. 15(*a*), *Tyroglyphus longior*, a mite. No. 15(*b*), *Trichomonas vaginalis*. No. 16 (*a*), Egg of *Dioctophyme;* (*b*) *Echinococcus* hooklets; (*c*), *Schistosoma* egg; and (*d*), *Wuchereria bancrofti* embryo.

In certain general diseases, as smallpox, purpura, and leukaemia one may expect haematuria. In the tropics it is a finding in yellow fever (asthenic stage) and in plague as well as in bilharziasis and the haematochyluria of filariasis.

Haemorrhage from the kidneys may arise from malignant growths, especially hypernephroma, or from benign ones, as papilloma of the pelvis. Blood in the urine may assist in differentiating a Dietl's crisis from hepatic colic.

Tuberculosis, whether of kidney or bladder, is apt to show haematuria. Drugs, especially turpentine, methenamine, carbolic acid and cantharides, give rise to haematuria. Renal varix in healthy individuals may cause haematuria.

It must be remembered that often no cause for haematuria can be found.

Haematuria occurs not only in acute nephritis but in some chronic cases. Chronic passive congestion is associated with red cells in the urine and renal infarctions from endocarditis may cause their appearance.

Stone in the pelvis of the kidney is an important cause. In the bladder we have as chief causes (1) tumors, malignant or innocent, and (2) calculus. In tumors the bleeding is not markedly controlled by rest as is that from stone.

In the urethra, gonorrhoeal inflammation, especially when near the neck of the bladder, and traumatism may be associated with haematuria.

Haemoglobinuria.—The two diseases one always thinks of in connection with haemoglobinuria are blackwater fever and paroxysmal haemoglobinuria.

Bacteriuria.—Bacterial infections of the genito-urinary tract are associated with more or less *pyuria*. Kidney infections are now recognized as most often derived from the blood stream rather than from extension from portions of the tract lower down.

The most important haematogenous bacterial infections of the kidney are, in order of frequency, those of the colon bacillus, staphylococci, streptococci, *Gonococcus*, *Proteus*, typhoid and paratyphoid. Renal tuberculosis is generally haematogenous rather than an ascending infection. *A pyuria without bacteria easily recognizable microscopically or culturally should suggest tuberculosis.* Very important is the differentiation of the pyuria of cystitis and pyelitis. Of course bladder irritation may follow pyelitis, and a suggestion of cystitis is often the first sign of renal tuberculosis. Prostatic obstruction often suggests cystitis.

The use of two sedimentation glasses will differentiate pus from the urethra from that from bladder and pelvis of kidney. If the urine in the first glass alone is turbid it shows urethral pus. With cloudiness of the contents of the second glass the problem is more difficult because it is almost impossible to differentiate a bladder involvement from a renal one by microscopic examination. Cystoscopy is necessary. Of course, some authorities attach importance to finding pelvis epithelium, others to the acid urine characteristic of pyelitis and the alkaline one of cystitis. Again it is often noted that the albumin content of the urine of pyelitis is far greater than that from cystitis. This is true for pyelonephritis but does not hold for simple pyelitis. By having the patient pass each voiding of urine during the 24 hours into separate bottles and then noting the amount of pus in each we may suspect bladder pyuria when the amount of pus sediment is the same in each bottle. In renal pyuria discharge of pus is intermittent so some bottles show much pus, others but little.

Pyelitis, usually a colon infection, must always be thought of in vague disorders of children and pregnant women.

Renal stone and renal tuberculosis often give similar symptoms but the X-ray and animal inoculation test should differentiate.

NOTE.—For discussion of albuminuria, glycosuria etc. and chemical tests see appendix.

	Amount and character of urine	Sp. gr.	Albumin	Sediment	Etiology	Special features
Acute nephritis.	Diminished; color dark.	High 1025 to 1030.	High 0.5 % and more.	Large amount. Hyaline, granular, epithelial and blood casts. Renal epithelium. Red and white blood cells.	Infectious diseases. Chilling. Poisons.	Sudden onset. Oedema often marked, especially of face. Mild or even severe uraemic symptoms. Pulse tension increased but heart not hypertrophied.
Ch. parenchym. nephritis.	Normal or diminished.	Moderately high 1020 to 1025.	Large amount. 0.5 to 2 %.	Abundant. All kinds of casts including fatty and waxy. Red blood cells. Much fatty epithelium.	Following acute attack. Alcohol, syphilis, malaria. T. B. suppuration.	Marked oedema. Uraemia common. Hypertrophied left ventricle. Blood pressure increased.
Ch. interstitial nephritis.	Greatly increased (2000 to 4000 cc.). Clear. Color pale and bright.	Low 1005 to 1010.	Trace; rarely exceeding 0.1 %.	Very slight. Very few hyaline and finely granular casts. Red blood cells in acute exacerbation.	Heredity, gout, syphilis, alcohol, lead, arteriosclerosis.	No oedema until later. High blood pressure (200 to 250). Cardiac hypertrophy; often uraemia and albuminuric retinitis.
Passive congestion.	Diminished; high color.	High 1025 to 1030.	Small amount.	Sedimentum lateritium; occasional hyaline casts. Red and white blood cells. Renal epithelium exceptional.	Ch. heart and lung disease.	No uraemia. Symptoms attributable to heart. Frequently urobilin.
Pyelitis.....	Normal, slight turbidity. Often haematuria.	Normal.	Slight.	Abundance of pus cells. Caudate epithelium often in clusters. At times red blood cells.	Bacterial infection ascending or descending. Stone.	Reaction of urine acid. No tenesmus.
Cystitis.....	Normal but very turbid.	Normal.	Slight.	Very great abundance of pus cells. Much mucus and bacteria.	Bacterial infection.	Reaction of urine alkaline or very faintly acid. Tenesmus.

CHAPTER XXXI

THE FAECES

It is advisable to examine a stool macroscopically before taking up the microscopical examination. The mucus shreds or casts of the bowel characteristic of mucous colitis or membranous enteritis may lead to the correct diagnosis of obscure abdominal pain. Pus in stools may often be recognized without the aid of the microscope.

The normal stool is sausage-shaped and soft. Neither the special form of scybalous masses called sheep pellets nor the pencil-like nor the tape-like excrement prove the existence of stricture of the intestinal lumen although suggestive of such a condition. The mucus of bacillary dysentery is opaque and grayish from the great number of pus and phagocytic cells. It is well to remember that Charcot-Leyden crystals, which are practically always absent from bacillary dysentery stools, are not infrequent findings in amoebae-containing stools; of course, these crystals appear in other intestinal parasite infections.

In obstruction of the common bile duct we have acholic, whitish, foul-smelling stools. If the putty color be due to bacterial change exposure to the air will restore the brownish tinge.

Sprue stools are white-wash to putty-colored, pultaceous, and filled with air bubbles. The amount is excessive.

Fatty stools are best examined microscopically.

Test Diet.—If the purpose of the examination is to determine the digestive power of the alimentary tract for proteids, carbohydrates, or fats, it is best to use a test diet, as that of Schmidt and Strasburger.

Prior to using this test diet, one should familiarize himself with the macroscopic and microscopic appearances resulting from such a diet in a normal person; standards are then at hand by which to judge of variations from the normal. The examination of the faeces of persons on ordinary and specifically undetermined articles of diet is very unsatisfactory when the state of digestion of muscle fibers and the question of fat digestion are at issue.

Before examining the faeces resulting from a test diet, wait until the second or third day so that the faeces of previous diets may have passed out. A charcoal powder taken before commencing the diet serves as an indicator.

Diet.—Breakfast, 7 A. M., bowl of oatmeal gruel (40 grams oatmeal, 10 grams butter, 200 cc. milk, 300 cc. water). Also one very soft-boiled egg (one minute) and 50 grams zwieback. In the forenoon, 500 cc. of milk.

For dinner, 2 o'clock, chopped beef broiled very rare (125 grams with 20 grams butter poured over it). Also a potato puree (200 grams mashed potato, 50 grams milk, 10 grams butter). Also ½ liter of milk and 50 grams zwieback.

For supper, 7 o'clock, the same articles as for breakfast.

This detailed diet may be varied to suit circumstances as regards interchanging meals. Furthermore, the milk may be taken in the form of tea or cocoa or cooked with the other food. Even a small amount of wine may be permitted. The diet taken, however, should absolutely conform to the following requirements: (1) The taking of ¼ pound chopped beef, a portion of which should be half raw; (2) the milk taken should amount to about a quart; (3) about 4 ounces of bread or toast and from 4 to 8 ounces of potato puree should be eaten daily.

The detailed diet contains about 110 grams albumin, 105 grams fat and 200 grams carbohydrates and has a fuel value of 2247 calories.

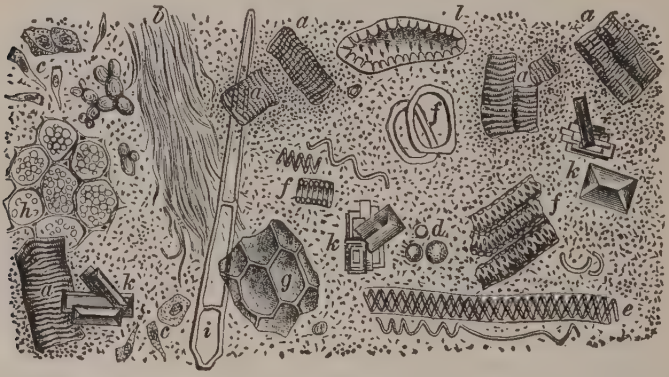


FIG. 196.—Microscopical constituents of faeces. (*v. Jaksch.*) *a*, Muscle fibers; *b*, connective tissue; *c*, epithelium; *d*, leukocytes; *e*, spiral cells; *f*, *g*, *h*, *i*, various vegetable cells; *k*, "triple phosphate" crystals; *l*, woody vegetable cells; the whole interspersed with innumerable microörganisms of various kinds.

The stool is best collected in quart fruit jars and examined as soon after evacuation as possible. The wooden spatula-like tongue depressors are well adapted to handling the specimen.

Having familiarized one's self with the degree of digestion of muscle, starch, and fat in a normal person, we are in a position to judge of the digestive and assimilative powers of a patient.

Macroscopical examination.—The first part of the test is the macroscopical one. For this grind up a faecal mass of ½ to 1 inch diameter in a mortar, gradually adding water until it has the consistence of a broth. About 0.5 cc. of this emulsion should now be squeezed out between two slides and studied against a dark surface and also when held up to the light. The normal stool gives a rather uniform brownish homogeneous layer. Connective-tissue remnants (indicative of gastric derangement) show as whitish fibers; undigested muscle tissue remnants as reddish-brown

splotches; fat particles as whitish-yellow clumps. Potato remnants appear like sago grains and mash out easily like mucus. Mucus is best noted in the faecal mass before making the emulsion.

Microscopical examination.—In the microscopical test of this emulsion: We judge of muscle digestion by the intactness of the striations. If a muscle remnant is only a homogeneous yellowish particle, it shows satisfactory digestion. If it is rectangular, with well defined cross striations, it shows poor digestion for meat (azotorrhoea). A loopful of faeces should be smeared into a drop of Gram's solution for starch-digestion determination. Normally there should be no blue-staining starch granules.

In the microscopic examination, epithelial cells are generally found to be more or less degenerated.

Triple phosphate crystals are frequently observed in faeces as may also be crystals of various calcium salts. Charcot-Leyden crystals are rather indicative of helminthiasis.

FIG. 197.—*A*, neutral fat; *B*, fatty acid liberated by acetic acid; *C*, soaps; *D*, fatty acid crystals. (*From Hawk's "Physiological Cehmistry."*)

Soaps are gnarled bodies everted like the pinna of an ear, while soap crystals are comparatively coarse and do not melt on application of gentle heat as do the more delicate fatty acid crystals. The fatty acid crystals also dissolve in ether which is not the case with soap crystals. Neutral fat is in round or irregular globules. The best stain for fat is Sudan III (saturated solution of Sudan III in equal parts of 70% alcohol and acetone).

Mix up the faeces with dilute a'cohol (50 to 70%) and then add a drop of the above solution and apply a cover glass quickly. The fat globules show as orange or golden-yellow bodies.

By rubbing up a small portion of the faeces in 36% acetic acid, apply ng a cover-glass and heating over a flame until the preparation shows bubbles, we convert the soaps and other fat combinations into free fatty acids which show as more or less

numerous highly refractile bodies showing a crystalline structure as the preparation cools By practice one learns the amount of such globules to expect with different fat contents in stools.

Steatorrhoea, or the presence of fat in abnormal quantities in the faeces, is shown by the appearance of the pale, bulky, greasy stools as well as in the microscopical examination.

Average for normals in 1 gram dried faeces:

Total fat	.225 mg.	(22.5%)
Total fatty acid	86 mg.	(37.9% of all fat)
Total soap	74.7 mg.	(33.4% of all fat)
Total neutral fat	64.4 mg.	(28.5% of all fat)

In normal cases the only fat elements recognizable are yellow calcium or colorless soaps. In sprue from 25 to 30% of the fat ingested appears in the stool while the stool of pellagra, a disease possibly confused with sprue, shows only about 5% which is the normal figure.

As quantity of fat increases (as say 500 to 600 mg.), droplets of neutral fat appear with or without increase in number of soap masses. Also needles and splinters of fatty acid and soaps appear. Much connective-tissue débris shows defect in gastric digestion, as only the stomach digests connective tissue.

Chemical Tests of the Faeces and Their Significance.—Certain of these tests are given in the Appendix (p. 747). Schmidt's test serves as a rough measure of *digestive power* for proteins and carbohydrates. A distinct evolution of gas within 12 hours indicates defective starch digestion, and the faeces mixture in the bottle will be acid to litmus. A delayed production of gas (after 24 hours) is evidence of albuminous decomposition, and the mixture will be alkaline. Urobilinuria may indicate impaired functional power of the liver, and indicanuria is usually due to stasis in lower part of small intestine.

Questions relative to *biliary excretion* and *pancreatic function* are best determined by examination of the duodenal contents. Findings in the faeces are a very crude and unsatisfactory index of pancreatic activity. Normally, bile pigments reach the anus in the form of hydrobilirubin, and bilirubin is not found below the ascending colon. The finding of bilirubin or biliverdin, characteristic of abnormal intestinal decomposition in infants, is abnormal, and at least indicative of increased intestinal peristalsis.

Occult blood is chiefly sought in cases of carcinoma, ulceration of the gastro-intestinal tract, etc. Extraneous sources of the blood, food, mouth, nose, lungs, vagina, etc., as well as interfering substances, must be excluded. A milk diet, or at least one without meat or green vegetables, is indicated for two to three days previous to test, and medication should be stopped.

Diastase is an amylolytic ferment whose activity is considered to be normally under the inhibitory control of the internal secretion of the pancreas, and whose function is concerned with the production of the blood sugar from the glycogen of the body. It is found in the body fluids and excretions. An impaired kidney function may cause its retention in the blood, and thus be the etiological factor in the hyper-

glycaemia of such cases. Its precise diagnostic significance is still debatable but very high values, especially in the blood, should be considered confirmatory of impaired pancreatic function. Constipation decreases and diarrhoea increases the amount excreted in the faeces, but low values occur with obstruction of pancreatic duct. Determination of its amount in blood and urine is of more value than in faeces. Some use the blood determination as an index of dietetic efficiency in diabetes mellitus. The urine determination is said to be valuable in the diagnosis of acute pancreatitis if diabetes and nephritis are excluded. It is also of some service as an index of kidney function.

Intestinal Protozoa in Stools. *Amoebae.*—A very practical way of obtaining material to examine for the presence of amoebae is to pass a rectal tube or a piece of drainage tube with fenestrations into the bowel. Amoebae may be found in the mucus filling the perforations in the tube. Walker advises against the use of salts in examinations for amoebae. In examining the stool microscopically, it is well to color the drop of faeces, which is to be covered with the cover glass, with a small loopful of 0.5% solution of neutral red. If diluting fluid is used, it should be salt solution, and not water. The neutral red tinges the granules of the endoplasm of amoebae and flagellates a very striking rose-pink color, thus differentiating them from vegetable cells or body cells. The iodine-eosin stain or simply emulsification of the faeces in Gram's iodine solution is more frequently used.

Whether examining the thin faeces or the mucus particle, it is well to reserve report on amoebae or flagellates until motion is observed. Encysted protozoa give an experienced examiner the surest method of recognizing the distinct eight nucleated cyst of *E. coli* and the one, two or four nucleated cyst of *E. histolytica*. In the mucus of bacillary dysentery stools, large intact phagocytic cells are frequent, which may be mistaken for vegetative amoebae.

Giardia.—Various flagellates, and in particular *Giardia*, may be responsible for diarrhoeal conditions which may cause rather serious symptoms.

Balantidium coli has been reported several times as the cause of dysenteric conditions. *Coccidia* are found in the faeces.

Isospora was not an infrequent finding in the stools of the soldiers at Gallipoli.

Bacteria in Stools. *Smears.*—When a smear preparation is desired, we may smear out a fragment of mucus and stain by Romanowsky's or Gram's method. The staining and morphological characteristics of the bacteria present have some diagnostic value—especially in the case of infants and young children Beautiful preparations may be made by mixing the faeces with water, then centrifuging for one minute. This throws down vegetable débris and crystals. Now decant the supernatant fluid, which holds the bacteria in suspension, and add an equal amount of alcohol. Again centrifuge, decant, and smear out and examine the sediment which contains the bacteria.

Simply taking a small mass of faeces and emulsifying it with a wooden toothpick on a concave slide in 70% alcohol—then, after the sediment settles, taking up a loopful with platinum loop from the surface and smearing out, gives a very satisfactory smear. Gram's

method, with dilute carbol fuchsin counterstaining, gives the best picture.

The Boas-Oppler bacillus may be found in the stools in this way. A Gram-stained stool normally shows a great preponderance of Gram-negative bacilli and such a finding in a measure excludes cancer of the stomach. Organisms which are Gram-positive as well as the Boas-Oppler bacillus are: (1) Lactic acid bacilli—these show Gram-negative areas in the slender bacilli. (2) A type of bacillus similar in size to the colon bacillus but Gram-positive and noncultivable (found in acid stools). (3) Bacilli of the *B. subtilis* type.

It is very important to examine the faeces for tubercle bacilli. With children a diagnosis of tuberculosis may be made in this way when the sputum cannot be obtained, the pulmonary secretion being swallowed. The preparation on the concave slide as described above should be stained for tubercle bacilli. Ulcerations in intestinal tuberculosis may show very numerous bacilli.

Culturing faeces.—To culture for typhoid, dysentery, cholera, or other bacteria, take up the material in a tube of sterile bouillon and smear it out with a swab over a lactose litmus agar plate or an Endo or Conradi-Drigalski plate. Before streaking the plates they should be made very dry on the surface. This can be best done by pouring into a plate with a circular piece of filter paper in the lid and placing in the incubator for one-half hour to dry. The filter paper absorbs the moisture. Then inoculate the surface of the plate with the faecal material. Selective cholera plating media are strongly alkaline.

Diarrhoeas of children.—In summer complaints of infants and children the organisms concerned are as a rule related to various strains of dysentery bacilli. Kendall in 293 stool examinations found the gas bacillus (*B. welchii*) in 22 cases. The gas bacillus produces intestinal disorders which are not benefited by lactose but by buttermilk (lactic acid bacteria). For diagnosis, a loopful of the faeces is emulsified in a tube of sterile milk or litmus milk. The emulsion is heated to 80°C. and held at this temperature for twenty minutes. After incubation for eighteen to twenty-four hours, preferably anaerobically, we get (1) a shreddy disruption of the casein, (2) the smell of rancid butter and (3) fully 80% of the casein is dissolved. Smears show short thick Gram-positive rods with slightly rounded ends. *B. subtilis* is sometimes found but does not give a rancid odor or the strong disruption of the clot.

Intestinal Helminths.—It is the faeces we examine either for the parasites or for their ova in connection with practically all the flukes, except the lung fluke and the bladder fluke; for intestinal taeniases and for practically all the round worms, except the Filarioidea.

Bass has recommended that faeces which have been made fluid be centrifuged and the supernatant fluid containing vegetable débris poured off. The sediment contains hookworm eggs. Then pour on sediment a calcium chloride solution of sp. gr. 1050. Again centrifuge and decant. Next add calcium chloride solution of a sp. gr. of 1250 and centrifuge. This brings to the surface the hookworm eggs which may be pipetted off. As a rule, the finding of hookworm eggs is very easy without such a technique. The eggs of *Trichostrongylus* greatly resemble those of

hookworm but are larger, 73 to 91μ long. In perfectly fresh faeces *Strongyloides* are present as worm-like embryos while hookworm gives only two- to four-segment eggs. See Barber's method under hookworms.

The larvae of various insects may at times be detected in the stools, as well as certain acarines (cheesemites, etc.).

The test for occult blood is indicated in helminthiases as well as in the conditions in which it is usually employed.

In the tropics, the examination of the faeces vastly exceeds in value that of urine and is possibly more important than blood examinations.

Gall Stones.—Gall stones are usually recognized by their facetted appearance The stool should be examined for two weeks following an attack of hepatic colic and the faeces should be rubbed up in water and passed through a sieve. A concentric arrangement of layers is usually noted on fracturing a gall stone.

False gall stones are usually masses of fats, soaps or vegetable material. It is well to dissolve the suspected mass in hot alcohol and examine for cholesterol crystal, upon evaporation of the alcohol.

For chemical identification, see p. 747. At times enteroliths are mistaken for gall stones. These are shells of inorganic salts covering inspissated masses of faeces or seeds, etc.

CHAPTER XXXII

BLOOD CULTURES

Clinically, the most important examinations of the blood for parasites is for the presence of various bacterial infections and for certain blood protozoa and also filarial embryos.

Culturing of typhoid and allied organisms.—A method of culturing blood, especially for the detection of typhoid or paratyphoid bacilli, is by the use of the bile media of Conradi. Test tubes are filled with 7 to 10 cc. of 1% peptone ox bile, or ox bile alone, and the medium is sterilized in the autoclavé. It is good practice to place the syringe in a plugged test tube containing salt solution, w th the needle unscrewed. After autoclaving, the sterile syringe can be taken to the bedside in the test tube. Using a wide test tube, a forceps can be sterilized at the same time and used to attach the needle to the barrel of the syringe.

By using a piece of glass tubing into which the needle is inserted we may sterilize the syringe easily in the test tube. The glass tubing prevents the steel needle from coming in contact with the glass of the test tube and so prevents cracking the test tube.

The skin should be scrubbed gently with green-soap solution and water for about three minutes. The skin of the area to be punctured should then be sterilized by the gentle application of Harrington's solution (not scrubbed) for one-half minute, and should then be washed with sterile water. It appears to be safe simply to scrub the area with 70% alcohol for one or two minutes. Application of pure carbolic acid on a gauze wad for a few seconds followed by neutralization with 70% alcohol gives satisfactory sterilization. The usual method of sterilizing skin for taking blood or inoculating vaccines is simply to smear the site of entrance for the needle rather heavily with tincture of iodine. A tourniquet is now applied to distend the vein, and the needle, beveled side up, is inserted in the direction of the venous flow. Withdrawing 5 to 10 cc. of blood, we loosen the tourniquet (otherwise the blood may flow from the puncture) then withdraw the needle, and force out about 0.5 cc. into the first bile tube, about 1 cc. into the second, and 2 or 3 cc. into the third. It is well to reserve some of the blood for Widal tests.

The bile tubes are now incubated for ten to twelve hours and then transfers are made to bouillon tubes. These bouillon tubes can be used in six to eight hours for testing the organism against known typhoid or paratyphoid sera. Test tubes containing 10 cc. of ordinary bouillon with 1% of sodium citrate are as satisfactory as bile media.

Some prefer a 2% sodium glycocholate in bouillon while others use a 2% solution of ammonium oxalate in bouillon for blood cultures.

Some prefer to streak plates of lactose litmus agar with material directly from the bile tubes instead of inoculating the bouillon tubes. Staphylococci present as contaminants, or other organisms derived from the blood in cases of septicaemia, are easily differentiated from the typhoid group.

Schottmuller adds 1 to 3 cc. of blood to liquefied agar at 45°C., and after mixing pours into plates. The standard method formerly was to add the blood to an excess of bouillon (1 to 5 cc. of blood to 100 cc. or more of bouillon).

By using the bile media, we can take the blood from the ear in typhoid cases if preferred. Then if chance staphylococcic contamination occurs, such colonies are readily differentiated from typhoid ones by the pink color on lactose litmus agar.

Routine blood culturing.—The following method of obtaining blood for culture has been used in our laboratory with good results. However, we recognize that the use of the hypodermic syringe is less liable to yield contaminating organisms.

A stout hypodermic needle is attached to about 6 inches of rubber tubing which in turn is pushed over a downward-bent glass tube which passes through a doubly perforated rubber stopper. A second glass tube, which also passes through the stopper, is bent upward to be attached to a second piece of rubber tubing for use in suction by the mouth. We keep on hand sterilized pieces of glass tubing packed with a little cotton to prevent the possibility of any saliva getting into the system. These are mouth pieces and are inserted after the tubes and stopper are sterilized. The glass tubes project about ½ inch below the under surface of the rubber stopper; above, they are about 2½ inches in length including the bent arm. This system of tubing and stopper is readily sterilized. As a receptacle for the blood we employ Erlenmeyer flasks of 100 cc. capacity, containing 10 to 25 cc. of salt solution with 1 or 2% of sodium citrate, for prevention of coagulation. These citrated salt solution flasks are plugged with cotton, sterilized and kept on hand ready for immediate use, so that we only have to sterilize the stopper and tubing by boiling and flame the neck of the flask when removing the cotton plug to insert the stopper of the system. By suction we can take any amount of blood desired. I usually count the drops of blood as they fall into the citrated salt solution allowing 16 drops to the cubic centimeter. In this way we may take from 10 to 25 cc. of blood at the bedside and then later on in the laboratory, when it is convenient, inoculate various media from the flask. For plates add 2 or 3 cc. of this citrated blood to 6 or 8 cc. of melted agar at 45°C. The blood mixture can also be added to various sugar bouillons for fermentation reaction. Finally we place the receiving flask in the incubator and culture it as well as the other media. Some organisms do not develop in this citrated blood, possibly on account of the blood concentration. This can be avoided by adding 50 to 75 cc. of bouillon to the citrated blood remaining in the flask.

Of course in inoculating the various plating or sugar tubes from the flask there is some liability to contamination. This may be avoided by removing with a sterile pipette 10 to 20 cc. from the flask containing the citrated blood to carry out the inoculations instead of pouring out directly from the flask. See Fig. 12.

For culturing blood in septicaemic conditions, the blood should always be drawn from the vein and cultured either by mixing 1 to 2 cc. with melted agar and then pouring plates or by transferring to bouillon in excess (at least ten times as much bouillon as blood) and after eighteen to twenty-four hours' incubation plating out. For *Streptococcus* and *Pneumococcus* blood agar plates are to be preferred, the *Pneumococcus* giving green colonies with only a suggestion of haemolysis while haemolytic streptococci give an opaque colony with a distinct haemolytic zone surrounding it. We rarely culture blood anaerobically as the important pathogenic anaerobes (tetanus, gas gangrene and malignant oedema) do not tend to invade the blood stream during life. A case has been reported where the gas bacillus was isolated from the blood of a soldier with gas gangrene. There is an anaerobic streptococcus, *S. putridus*, which grows anaerobically on blood agar giving porcelain-white colonies without haemolysis. The cultures have a putrid odor. Not pathogenic for animals.

Inoculation of blood into animals.—A very useful procedure in the isolation of streptococci, pneumococci, plague and anthrax bacilli is to inject 1 to 2 cc. of blood into suitable animals. When injecting mice use only about 0.2 cc. subcutaneously at root of tail; more certain of results is the injection of about 1 cc. of the blood intraperitoneally. Streptococci, even from virulent human infections, are uncertain in their action on animals so that the failure to produce septicaemia in the mouse does not necessarily indicate that the organism is of slight virulence.

Bacteriaemia.—Warren and Herrick published a very important study of 134 cases of bacteriaemia. Bacteriaemia signifies the mere presence of bacteria in the blood without reference to symptoms, while sepsis denotes conditions due to invasion of the blood-stream by bacteria or their toxins with marked systemic reaction.

Of 25 cases with endocarditis, 22 died and 3 were unimproved. Of 55 cases of sepsis, 39 died and 10 recovered. In postpartum infections, 10 died and 1 recovered. In osteomyelitis, 5 died and 4 recovered. In otitis media, 2 died and 4 recovered.

Thirty-one cases were due to haemolytic streptococci of which 21 died.

Forty cases were due to *S. viridans* and 25 died. One case from *S. mucosus* died.

Of 39 cases with *Staphylococcus aureus* 22 died and in 3 cases of *S. albus* 2 died

Of 10 cases of *Pneumococcus* bacteriaemia 6 died. Six of *B. coli* infection gave 4 deaths; two of *B. influenzae* (Pfeiffer), 2 deaths; three of anaerobic streptococci, 2 deaths and two of *B. mallei*, 2 deaths.

Seven cases of mixed infection gave 6 deaths.

The ordinary leukocyte and differential count procedures were of very little value in prognosis.

The average white count in fatal cases of haemolytic streptococcic infection was 18,347 with 81% of polymorphonuclears while in cases recovering it was 18,742 and 85%, respectively. Fatal *S. viridans* infections gave an average white count of

15,976 with polymorphonuclear percentage of 78, while nonfatal cases gave 17,222 and 75%, respectively.

Of 25 cases treated with vaccines, 81% died; while of 47 under surgical treatment, 50% died and with 50 treated palliatively, 50% died.

General considerations.—Typhoid cultures are best obtained in the first week of the disease; after that time the Widal is the test of preference.

If a paratyphoid serum is not at hand for testing, it may suffice to inoculate a glucose bouillon tube or a Russell lactose glucose litmus slant; gas production indicates paratyphoid. This test should be applied when a very motile organism, cultured from the blood, does not show agglutination with a known typhoid serum. The possibility of obtaining anthrax and glanders in blood cultures should be considered.

In undulant fever it must be remembered that colonies do not show themselves for several days. Addition of blood to melted agar is a good procedure.

Blood for culturing typhoid or the paratyphoids may be taken with a Wright's tube from the ear or finger. Dipping the hand in hot water assists the flow of blood. The supernatant serum after centrifugalization should be pipetted off with a sterile pipette and reserved for agglutination tests while the clot is dropped into a bile tube. (Clot culture.)

Gastro-intestinal disturbance of obscure origin may be due to a *B. coli* bacteriaemia, in which case the bacilli may be cultivated from the blood stream.

Animal parasites found in blood.—The examination of the blood for the parasites of malaria, filariases, kala-azar and spirochaetal fevers has been discussed under their respective headings.

With trypanosomes from human trypanosomiasis, smears from gland juice or cerebrospinal fluid seem more satisfactory to examine than blood smears unless the blood is taken in 5 to 10-cc. quantities and centrifuged in sodium citrate salt solution.

A method in the diagnosis of trichinosis is to take 5 to 10 cc. of blood from a vein at the time of the migration of the embryos to the muscles (ten to twenty days). This is forced out into a centrifuge tube containing 3% acetic acid, and the sediment examined for trichina larvae.

CHAPTER XXXIII

THE STOMACH AND DUODENAL CONTENTS

Stomach Contents.—The examination of the stomach contents is still clinically unsatisfactory. There is much uncertainty as to the representative character of the specimens obtained, owing to the physiologic differentiation between the cardia and the pylorus, and the chemical variations in the chyme during successive stages of digestion; extragastric conditions may exert a profound influence upon gastric function; pathological material found in the stomach may have a source elsewhere, or be a secondary manifestation of disease elsewhere; and abnormal findings are rarely of such nature as definitely to indicate the diagnosis. Consequently there has been evident a tendency to omit gastric analysis in studying a case of gastro-intestinal disease. This is probably an unwise attitude. Although the information obtained from this source must always be correlated with history, clinical examinations and Roentgenologic findings, one should not therefore ignore the possibly valuable corroborative evidence it may furnish.

The prevailing skepticism as to its value has been due in part at least to the variable technique followed, and Rehfuss and Hawk have shown the necessity for standardization. They advocate the study of the gastric residuum, the use of the Ewald test meal, the fractional removal of stomach contents, the determination of motor as well as secretory characteristics, and repeated examinations.

But White and others have called attention to the marked variations in acidity between successive samples withdrawn at 30-second intervals with the position of the tip of the tube unchanged, differences that range from 4° to 36° for free HCl, and from 2° to 50° for total acidity. The practical value of fractional examination for the detection of secretory variations (acidity, ferments, etc.) is, therefore, very doubtful, particularly when we realize that change in position of the tip may add to the difficulty. Hence, unless one follows the Fitz routine, only gross variations in acidity are of clinical significance.

Normals for the residuum are given on p. 761. Abnormal findings are: A quantity of more than 100 cc.; total acidity of more than 70°, free acidity of more than 40°; less than 3 units (Mett) of pepsin; variation from 7 (Spencer) for trypsin; food retention; blood; pus; marked increase of gastric mucus. Increased volume without food retention may be due to pure vagotonia, psychic causes, or use of tobacco, but

it is usually the result of an organic lesion, frequently duodenal ulcer, less commonly chronic appendicitis or cholelithiasis. Too much food, alcoholism, acute illness, or atony may cause increased volume with food retention, but 98% of cases with 12-hour retention are due to organic lesion, such as dilatation or obstruction from any cause. High total acidity and free acidity occur with early stages of malignant ulcers and 40% of gastric ulcers. Crises, accompanied by deformity of bulb, are almost pathognomonic of duodenal ulcer. Low total acidity and low free acidity with persistent retention are almost always due to malignancy. Blood of gastric origin is found in 80% of cases of malignancy, but is less common than is usually supposed in ulcer.

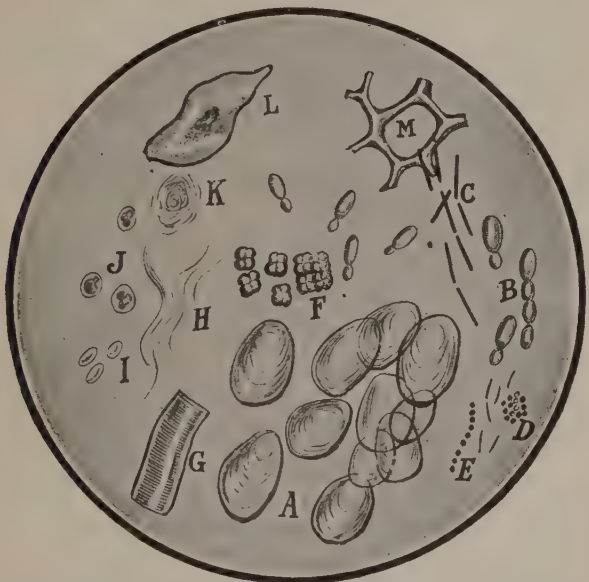


FIG. 198.—Microscopical constituents of the gastric contents. *A*, Starch cells; *B*, yeast cells; *C*, Oppler-Boas bacilli; *D*, staphylococci; *E*, streptococci; *F*, sarcinae; *G*, muscle fiber; *H*, mucus; *I*, red blood cells; *J*, leukocytes; *K*, snail-like mucus formations; *L*, squamous epithelial cells; *M*, cellulose. (*From Hawk's "Physiological Chemistry."*)

The microscopical examination of the sediment is not usually very informative. The addition of a dilute Gram's iodine solution is helpful, as muscle fibers, yeast cells, red blood cells, and epithelial cells are stained a golden yellow; starch granules are stained blue, while fats are unstained and show as highly refractile globules of varying size. One should seek mucus, epithelial cells, pus, blood, and bacterial colonies. The original epithelial cells may be digested and only the nuclei remain. The multilobed ones from leukocytes are quite characteristic. The buccal origin of material is indicated by squamous epithelium and by "salivary corpuscles," which closely resemble pus cells. An occasional columnar epithelial cell of gastric origin

may be found. In chronic gastritis, the picture of mucus entangling large numbers of cells is characteristic. It must be remembered, however, that strands of mucus entangling polymorphonuclear cells may be found in normal gastric contents. The pus cells that are free are the ones of significance.

The following signs of gastric cancer are useful in differentiating it from non-malignant dilatation: (1) Fragments of cancer tissue, especially cells showing mitoses. Such findings are very rare, however, and the diagnosis of the tissue is difficult. (2) The presence of flagellates in the early stages of cancer, as in the so-called anacid stage preceding the development of lactic acid. Since flagellates prefer an alkaline medium, they disappear when acidity due to lactic acid develops. (3) The presence of the Boas-Oppler bacillus. There are probably several organisms so designated. They are very large ($7 \times 1\mu$), Gram-positive, nonspore-bearing, arranged in long chains which stretch across the field of the microscope. They are aerobic, can be cultivated on media rich in milk or blood, and are lactic acid-producers. Heinemann thinks it probable that the Boas-Oppler bacillus, *Leptothrix buccalis*, and *B. bifidus* may be identical with *B. bulgaricus* (see under "Milk"). They should only be reported when present in great abundance and in long chains. (4) The absence of sarcinae and yeasts. Occurring in vomitus, these are indicative of a simple dilatation.

Observations of motility are important. The stomach normally empties itself of food in two to two and one-half hours. Hypermotility is associated with subacidity, achylia, scirrhous carcinoma with a funnel-shaped pylorus and antrum, "gastric chorea," and a very small percentage of duodenal ulcers. Slight delay is due to defective muscle action. Marked delay is the result of pyloric abnormality, hypertrophy, spasticity, or organic lesion such as malignant or benign tumors, ulcer, syphilis, or gastric ulcer on lesser curvature with organic biloculation. Extragastric tumors or adhesions may also cause delay.

Material of extragastric origin tends not to persist throughout the fractional examination. Swallowed muco-pus appears floating or in dense masses; that from the duodenum is associated with bile; that from the stomach is flocculent or intermixed with gastric mucus, and may be found only in the latter half of the series. Extragastric blood is usually aerated, floats in small, discrete, streaky flecks, and is associated with tissue elements not gastric.

Mere acidity values mean very little and normally vary widely, and it is very doubtful if the type of acidity curve upon fractional examination is more significant. There is no curve pathognomonic of any gastric condition. The normal pH for adults is 0.9 to 1.6; for infants, about 5.0.

Abnormal secretory variations with gastric mucus mean gastritis; with blood, they indicate erosion; occurring only with tryptic regurgitation or bile, the complex is almost pathognomonic of duodenal ulcer. With blood, pus, and mucus, all of intragastric origin, and bacteria, they signify erosion and infection, and the syndrome is found only in true infective gastritis, gastric carcinoma, gastric syphilis, and in the rare acute, phlegmonous gastritis. In the presence of gastric symptoms, secretory disturbances alone are found only as the result of extragastric causes.

Acidity is of but little aid in the diagnosis of ulcer. As regards acidity in cancer, the Mayo Clinic reported an average total acidity of 31°, free HCl of about 10°, lactic acid present in 43% of cases, and blood, occult or gross, in 73% of the cases. In a later and more recent analysis of 631 cases of carcinoma and carcinomatous ulcers relative to the presence of free HCl, they found, in round numbers, that 50% showed achlorhydria, while 0° to 20° was present in 15%, 20° to 40° (normal) in 20%, and 40° plus in 7%. The greater the extent of the malignancy, the greater seemed the tendency to lower value for free HCl, but location had no definite influence. Clarke and Rehfuss found that the Wolff-Junghans' protein curve shows a progressive separation from the acidity curve in cases of carcinoma, although the finding is not pathognomonic.

The Fitz routine, developed at the Mayo Clinic, comprises an ordinary supper, including meat; a few dried raisins about 12 hours before the test meal; nothing more by mouth until the test meal, which consists of 400 cc. water and 36 Gm. arrowroot biscuit, on the following morning. Remove total gastric contents one hour later by means of the Rehfuss tube. Examine for volume in cubic centimeters, represented by a, degrees free HCl, using Töpfer's reagent, represented by b; degrees total acidity to phenolphthalein, blood, and also microscopic elements.

His formula is $x = \frac{ab\sqrt{b}}{100}$, and normals are x and a of less than 600 and 300 respectively.

He reports 1039 cases in which the diagnosis had been controlled by Roentgen ray or laparotomy, and groups them into 4 classes, viz., (1) normal findings; (2) anacidity (without demonstrable free HCl); (3) hyperacidity without stasis (x of more than 600; a normal); and (4) hyperacidity with stasis (both x and a higher than normal). In the first group, 13% were in error, including duodenal and gastric ulcer, and, rarely, operable cancer. The second group suggests, but is not pathognomonic of, carcinoma in or near the stomach, and that lesion was present in 18%; more commonly, however, the conditions present were marked arteriosclerosis among the elderly, achylia gastrica in younger patients, pernicious anaemia, and chronic diarrhoea. Fifty per cent. of the third group had gastric or duodenal ulcer, chronic cholecystitis, or chronic appendicitis. Eighty-six per cent. of the fourth group had a demonstrable organic basis for the findings.

Vomitus, of course, can be examined and considered as a single, partial specimen of stomach contents. It usually contains a considerable admixture of buccal secretions.

Duodenal Contents.—The clinical significance of variations in this fluid is even less well established than for stomach contents. We are also confused by the multiplicity of methods, as well as inconsistency in the interpretation of results. Methods need standardization, and a systematic study of large groups of normal and pathological individuals is indicated.

The duodenal contents as withdrawn from a normal person, may be described as a viscid fluid, alkaline to litmus, usually clear or slightly turbid, pearly-gray in color or tinted by bile, and usually with only a slight sediment. The pH (three to four hours after meals) fluctuates around 7.0, with greater variations to the acid then to the alkaline side. The technique of examination is considered on p. 746.

The color of the specimen is as satisfactory a test for bile as the usual chemical tests. Color will change rapidly, and the golden-brown tint due to bilirubin is usually soon replaced by a green (biliverdin).

The examiner must anticipate obtaining not only the true intestinal secretion, but also a variable addition from gastric, biliary, and pancreatic sources. Moreover, the tube constitutes a foreign body in the mouth, oesophagus, and stomach, and probably causes considerable salivary and gastric secretion that will reach the duodenum. Under such conditions, considerable latitude must be allowed for any characteristic. At present, it is better to reserve judgment, and only offer what appears to be a reasonable opinion as to diagnosis, considered in conjunction with the clinical picture and X-ray findings.

As regards the biliary tract, consider the sample showing bile, and especially the response to a cholagogue, such as HCl. Pancreatic secretion will be indentified by its ferments. The sample secured during the resting, fasting state is the desirable one relative to the duodenum.

The pathological significance of turbidity due to amorphous material (a quite common finding) is probably overrated. Inflammatory conditions would be accompanied by increased viscidity, living bacteria, desquamation, and probably abnormal amounts of protein (Wolff-Junghan's test p. 745), and McNeil considers a turbidity in the 1–80 tube as normal, but one in higher dilution as abnormal.

The enzymes will manifest much variation as a whole and individually, the strength of any one not necessarily being an index for the others. They should be considered in relation to the intake of food and its nature. Low values of trypsin, probably the enzyme most constantly found, are usually associated with low values for amylase and lipase. Lipase is almost as constantly present as trypsin; amylase by no means so. Low "alkalinity" is usually associated with low tryptic and amylolytic power, but would appear to have much less effect upon lipolytic activity.

In the microscopical examination, the use of Gram's iodine solution as for stomach contents is convenient. It should, however, follow examination of an unstained specimen, as thus the bile-stained elements are easily detected and their association with the biliary tract established. Search should be made for epithelial cells, red blood cells, leukocytes, mucus, bacteria, parasites, food remnants, digestion products, etc. It must be borne in mind that, powerful enzymes being present as a rule, food material rapidly disintegrates, and digestion products, such as fatty acids, soaps, etc., may be encountered. Epithelial cells may come from so many sources, that caution should be observed in stating their origin and in drawing deductions from their presence. Squamous epithelial cells, cells from the biliary tract, and nuclei seem to be more resistant to digestion then are other elements. Normally, cells and living bacteria are few. We have found flagellates and 2-celled ova of the hookworm in the contents.

The bacterial flora is interesting, and often of etiological importance. In a series of 22 routine cases, we obtained a growth with 50%, the organisms being staphylococci with, occasionally, Gram-positive bacilli. Kelly, in a bacteriological study of 413 cases of biliary-tract operations by Deaver, found 55% sterile, but, in the acute cases, living bacteria were almost constantly present. *B. coli* was present in 30%, *B. typhosus* in 7%, *Staph. pyogenes* in 2.9%, and streptococci in 0.2%; other organisms were more rarely found. Gessner reported the rather constant presence of *B. lactis aerogenes*. Typhoid carriers are most surely detected by culturing the duodenal contents.

CHAPTER XXXIV

EXAMINATION OF PUS

Pus may be collected for examination either (1) with a platinum loop, (2) with a sterile swab, (3) with a bacteriological pipette or (4) with a hypodermic syringe.

It is always well to make a smear and stain it by Gram's method at the same time that cultures are made. The Gram stain gives information as to the abundance of organisms in the pus and as to the probable findings in the culture. Pneumococci and streptococci are differentiated from the staphylococci in this way without the necessity of more or less extended cultural methods.

Smears from material examined for gonococci may show Gram-negative diplococci which, however, do not generally have the morphology of the *Gonococcus*. They are furthermore extracellular.

The *M. catarrhalis* has been reported from urethral smears though very rarely. Diphtheroid organisms are not uncommon. Gram-positive cocci are rather common in smears from discharges of chronic gonorrhoea.

When autogenous vaccines are to be made, the isolation of the exciting organism is necessary. This is best done by streaking the pus, taken up with a sterile swab and emulsified in a tube of bouillon, over the surface of an agar plate. Practically as convenient and providing a more nutritious medium is to smear the material on a loop or swab over the surface of a blood-serum slant, then to inoculate a second tube from the first without recharging the loop or swab, and so on until three or four tubes are inoculated. Isolated colonies should be obtained in a third or fourth tube.

In examining blood-serum slants inoculated with purulent material, always examine the water of condensation for streptococci. I now use blood agar.

A bacteriological pipette is very useful when pus is to be sent to a laboratory; the tip can be sealed in a flame and the cotton plug at the other end insures the noncontamination of the contents. The material may be drawn up either with the mouth or with a rubber bulb.

The hypodermic syringe is very useful in puncturing buboes, etc., especially in plague. A small pledget of cotton on a toothpick dipped into pure carbolic acid and touched to a spot over the bubo, the escharotic action being arrested with alcohol after about thirty seconds, makes a sterile anaesthetic spot at which to introduce the needle of the syringe. It must be remembered that when plague buboes begin

to soften, the plague bacilli may be replaced by ordinary pus organisms. The pus from wounds infected with anaerobes is usually very foul. The most important anaerobe in the discharge from gas gangrene wounds is *B. welchii.*

War wounds.—In a study of the aerobic bacterial flora of war wounds Lawrence found that more than 80% of the discharges from such wounds showed streptococci, which especially flourished in deep pockets, staphylococci replacing them in shallow wounds. Gram-negative bacilli were present in 95% of smears. Of these, *B. coli* was present in 50% of cases. *B. cloacae*, *B. rhinoscleromatis* and *B. alkaligenes* were isolated in from 10 to 20% of wounds. As the suppuration continued *B. pyocyaneus* became frequent. The aerobic spore-bearers, especially *B. subtilis*, *B. mesentericus*, *B. vulgatus*, *B. megaterium*, *etc.*, were observed in about 20% of wounds but disappeared promptly under treatment. The combination of aerobes and anaerobes in a wound makes conditions more favorable for the anaerobes. Wounds contaminated with *B. fusiformis* do badly. In carrying out the Carrel treatment, daily smears are made of the discharge from the most markedly suppurating part of the wound. These are stained by Gram's method. The wound is not closed until the bacteria are so reduced in number that only one or two organisms can be found in each oil-immersion objective microscopic field.

The pus from the necrotic center of climatic bubo is sterile.

It is remarkable how frequently we get pure cultures from abscess material. In purulent material from abdominal abscess we are apt to obtain mixed cultures, especially the colon bacillus and *B. pyocyaneus*, in addition to ordinary pus organisms.

When it is a question between streptococci and pneumococci, it is well to inoculate a mouse; finding the capsulated pneumococci at the autopsy makes the diagnosis.

Animal inoculation also is necessary in plague and glanders, and possibly anthrax. When tetanus is suspected, it should be examined for as described under Tetanus. Tuberculosis should be identified by inoculating a guinea pig, as well as by acid-fast staining and culture, if there is any doubt as to the nature of the material.

The black or yellow granules of madura foot, as well as those of actinomycosis, should be examined as recommended in the section on fungi.

Amoebae, coccidia, and larval echinococci may be found in purulent material, as may also various other animal parasites, as fly larvae, sarcopsyllae, etc.

The pus from an amoebic abscess of the liver is as a rule sterile when cultured, and the examination at the time of operation or exploration frequently shows an absence of amoebae as well as of bacteria; but two or three days later amoebae may be found in the pus draining from the abscess cavity.

Flukes, round worms and whip-worms may as a result of their wandering from the intestinal lumen cause abscesses.

Serious ulcerations may follow infection with the Guinea-worm.

Abscesses of filarial origin are to be thought of.

Focal Infections.—In recent years our attention has been directed to the importance of certain localized bacterial foci which may extend through blood or lymph channels and give rise to various systemic or localized diseases. Most important of these diseases are various types of arthritis together with endocarditis, myocarditis and pericarditis. Next in importance are renal infections, chiefly of the glomerulonephritis type.

Cholecystitis, appendicitis, pancreatitis and various skin lesions may also have origin in a focal infection. The primary foci may be localized in any part of the body but those seated in the tonsillar, peridental membrane, nasal and accessory sinus tissues are the most common and important. Focal infections of the genito-urinary tract may also give rise to generalized conditions as is also true of such foci in the alimentary tract. In the tonsils we should particularly examine the material of crypts for various streptococci and likewise the bacterial flora of tooth abscesses or pyorrhoea alveolaris.

Table Showing Number of Times Each Focus Was Considered a Probable Source of Infection in a Series Studied by Billings and Associates

	No.		No.
Tonsil	336	Prostate and genito-urinary tract	24
Teeth	136	Gall-bladder	3
Sinus	12	Enterocolitis	2
Bronchi	5	Appendix	1
Uterus and tubes	12	Middle ear	1

Foster, in the report of a clinical study, gives as his opinion that nephritis not due to well recognized specific causes has its origin in infection, and that removal of the infective focus offers the one reasonable expectation of cure. He names the following localities in which the focus may be situated, arranged in order of importance: (1) Teeth; (2) tonsils; (3) sinuses; (4) gall-bladder, and (5) prostate.

In studies of the bacterial flora of excised adenoids, the presence of pathogenic organisms was demonstrated in every specimen of material taken from the crypts or from between the folds. Haemolytic streptococci and pneumococci were present in half the cases, and the Pfeiffer bacillus in almost as many. Diphtheria bacilli were found in about 10% of the adenoids cultured. Removal of the organs effected a reduction in the pathogenic flora of the naso-pharynx, but not its disappearance. In view of this persistence of pathogenic bacteria after removal of foci of disease, and the demonstration of their presence in a considerable percentage of normal children, it is evident that they are not necessarily associated with obvious disease or a surgical degree of hypertrophy.

Daland calls attention to the value of the combination of lymphocytosis, decrease in polymorphonuclear cells and leukopenia as an indication of the presence of a focus of infection within the body. The signs appear to be of value in the case of a focus anywhere, but are particularly reliable in indicating chronic apical infections of the teeth showing streptococci of the haemolytic and viridans types.

In a table prepared by Billings and associates, the organisms obtained were:

Focus	No streptococci found	Only streptococci found were haemolytic streptococci	Only streptococci found were non-haemolytic or green-producing streptococci	Mixed streptococci	
				Non-haemolytic predominating	Haemolytic predominating
Tonsil	0	20	34	150	50
Teeth	16	8	63	39	5
Sinus	3	1	5	3	0
Bronchi	0	0	0	4	1
Genito-urinary tract	6	2	10	0	1
Uterus and tubes	1	0	8	1	0
Gall-bladder	0	0	0	1	0
Appendix	0	0	0	0	0
Stool	5	1	1	0	0
Ear	0	0	0	1	0
Gland	6	1	19	1	0
Joint	6	1	5	1	0
Muscle	1	0	7	0	0
Bursa	0	0	1	0	0
Fibrous node	0	0	1	0	0
Blood	3	0	1	0	0
Total	47	34	155	201	57

NOTE.—In this study gonococci were found eight times in material from the genito-urinary tract, but never from other sources; while staphylococci were obtained four times, once from teeth and three times from the genito-urinary tract.

Elective localization.—In experiments with streptococci and pneumococci, Rosenow found that when organisms of exalted virulence were injected into suitable

animals there resulted haemorrhages and oedema of the lungs with bronchopneumonia, whereas, when organisms of lower grades of virulence were injected, endocarditis, arthritis, cholecystitis, gastric ulcer, myositis or iritis developed—these observations suggesting peculiar localizing tendencies in the infecting power of the organisms. The existence of such tropisms, or affinities, was further indicated by the results of intravenous injections into animals of streptococci isolated from certain foci. The bacteria exhibited in the experimental animals the same elective localization shown in the original hosts, producing lesions corresponding in type and location with those seen in the spontaneous disease. Rosenow found also a tendency of certain organisms isolated from apical abscesses of teeth or from tonsils to localize in the spinal cord.

Pleomorphism.—In the prosecution of his work on localization of bacteria in the spinal cord, Rosenow found that the streptococci under observation developed very small forms, capable of passing through the pores of test filters, which he regarded as identical with the globoid bodies of Flexner and Noguchi generally accepted as the cause of poliomyelitis. The development of these minute forms from streptococci of normal size is an example of pleomorphism, which may be defined as the assumption at different times of various distinct forms by a single organism or species. Pleomorphic changes are observed in young, actively growing cultures and are more or less permanent, usually persisting so long as the environment in which they arise remains unchanged.

Pleomorphic forms are to be differentiated from degeneration and involution forms which appear not uncommonly in old cultures and under unfavorable conditions in environment such as accumulation of waste products, unsuitable reaction or osmotic tension, and presence of harmful chemicals or specific antagonistic substances. If the change brought about is morphological, we term the atypical bodies involution forms: An example is the occurrence of bizarre shapes in plague strains grown on salt agar. If the strain studied loses permanently some morphological or chemical characteristic, we describe the new forms as degenerative: An example is the inability of anthrax to form mature spores when the strain is heated for several hours at 43°–44°C., or the inability of *B. prodigiosus* to produce its characteristic red pigment when cultivated at 40°C.

Pleomorphism is comparatively uncommon among pathogenic organisms, but a striking degree in the *B. tularense* is illustrated in Fig. 39, page 163.

Mutation of species.—It is still an open question whether or not true mutation of bacterial species has been observed. Rosenow, having brought about transformation of certain strains of bacteria, believes the changes wrought in their characteristics furnish instances of mutation, and the following examples of what he has accomplished may be cited: Twenty-one strains isolated as haemolytic streptococci were converted into *S. viridans*, and three into *S. viridans* and typical pneumococci, one of these into *S. mucosus* also. Seventeen strains isolated as *S. viridans* were converted into pneumococci and two of these into *S. mucosus* also. Eleven strains isolated as pneumococci have been made to correspond with haemolytic streptococci and seven took on the features of *S. viridans*. In order to ensure that pure cultures were used, cultures of each main variety that showed mutation were obtained from single organisms by the Barber technique. Rosenow states that the transformation effected was complete by every test known—morphology, presence of capsule,

fermentation powers, solubility or insolubility in bile and NaCl solutions, behavior toward broth culture filtrates (Marmorek), and the specific immunity response.

In judging of mutation, one must differentiate between temporary changes in secondary characteristics which revert rapidly to type when brought back to normal environment and those which constitute permanent inherited characteristics. Jordan considers that for bacterial variations to be accepted as evidence of true mutation they must appear suddenly without intermediate stages, must be irreversible and must not involve all the cells of a parent strain. It will be noted in Rosenow's experiments that new features, while at times arising suddenly, were often preceded by a pre-mutational stage, that they were reversible, that the new forms did not exhibit fixity and that apparently all the cells of the strains studied were involved.

While it cannot be denied that true mutation of species may take place, most of the instances adduced in support of the possibility may be classed as examples of adaptive modification arising from variation in environment and from development of latent characteristics.

CHAPTER XXXV

SKIN INFECTIONS

Cultural methods are as a rule to be preferred in the bacteriological examination of the skin.

These should be preceded by washing the surface to be examined with soap and water, in order to eliminate chance organisms which may have settled on the surface of the skin in dust or as a result of contact with material containing them. Scrapings are then made with a sterile dull scalpel, and the material obtained is emulsified in a drop of sterile water in the center of a Petri dish. A tube of melted agar at 42°C. is then poured on the inoculated drop and, by mixing, the bacterial flora is distributed throughout the plate. Of the colonies developing on such plates probably 80% will be found to be staphylococci, and of these the greater proportion will be staphylococci showing white colonies.

Occasionally the *aureus* or *citreus* may be isolated. Streptococci and colon bacilli are rarely found. The *Staphylococcus aureus* is the organism usually isolated from furuncles, circumscribed abscesses and carbuncles. Streptococci are the organisms to be expected in phlegmonous infections. Cold abscesses, which are frequently due to tuberculous infection, are, as a rule, sterile. Acne pustules may show staphylococci or the microbacillus of acne or both.

The *Bacillus acnes* is a short broad bacillus often showing a beaded appearance when stained by Gram's method. It is Gram-positive. According to Hartwell it grows readily on glucose agar when cultivated anaerobically (Wright's method). Colonies appear in four to five days.

Sabouraud's medium for its culture is: Peptone 20 grams, glycerin 20 grams, glacial acetic acid 5 drops, agar 15 grams and water 1000 cc. The bottle bacillus, which morphologically resembles a yeast, is considered to be the cause of dry pityriasis capitis. It may also be found in the comedones of children.

In the tropics, an organism which at times produces lesions similar to impetigo and again pemphigoid eruptions and at other times widespreading erysipelatous conditions gives cultural characteristics similar to *S. aureus*. It is probably only a virulent *aureus*. It has been described under the name of *Diplococcus pemphigi contagiosi*.

The *Staphylococcus epidermidis*, or stitch abscess coccus, is considered by Sabouraud to be the cause of eczema seborrhoicum.

It is in scrapings from the skin of lepromata that we find acid-fast organisms in the greatest profusion. In tuberculosis of the skin, the

tubercle bacilli are exceedingly scarce. Inoculation of a guinea pig will probably give positive results with the tubercle bacillus. The leprosy bacillus is nonpathogenic for experimental animals.

Anthrax and glanders cause skin lesions which can be surely diagnosed only culturally or by animal inoculation.

Plague bacilli may be isolated from the primary vesicles appearing at the site of the flea bite.

Tropical phagedaena is thought by some to be due to a sort of diphtheroid organism. The organisms of Vincent's angina may cause tropical ulcer. Herpes zoster has been reported by Rosenow as most probably due to a streptococcus with special affinity for the ganglia and posterior roots.

The skin diseases due to fungi are discussed under that section. Of the skin affections caused by animal parasites, ground itch (dew itch, foot itch), is the most important. This is a form of dermatitis due to the irritation set up by the hookworm larvae penetrating the skin of the foot and leg.

The *Tunga penetrans* (*Sarcopsylla penetrans*) or jigger (sand flea) is an important agent in ulcerations about the foot.

Certain acarines cause skin lesions, as is also the case with the larvae of certain flies.

The itch mite (*Sarcoptes scabiei*) is an important animal parasite of the skin.

The various lice, fleas and bedbugs are well recognized causes of skin irritation.

Filarial infections are also important especially the ulcers of the Guinea-worm, Calabar swellings of *Loa loa*, the cystic tumors of *Onchocerca volvulus* and the varicose groin glands and elephantiasis of *W. bancrofti.*

Leeches, as *H. zeylanica*, may cause serious ulceration.

Enterobius (*Oxyuris*) may cause a severe irritation about the region of the groin and inner surfaces of the thigh, and especially about the vulvar region of female children.

Gnathostoma spinigerum, a nematode with two lip-like structures and spine-like appendages covering its anterior one-third, has been found once in a tumefaction of the breast.

According to Lemaire, ten species of nematode larvae, other than those parasitic in man, may penetrate the skin, setting up a dermatitis. Not being human parasites they die out without infesting other tissues.

Plerocercoid larvae of Diphyllobothriidae have been found in the subcutaneous tissues.

Certain skin diseases, as Oriental sore, are protozoal in origin. The cutaneous lesions of uta or espundia are now known to be caused by a *Leishmania* as well as is Oriental sore. These affections in the Central and South American countries are now known as American leishmaniases.

For moulds and methods of examination for fungi see Chapter X.

CHAPTER XXXVI

CYTODIAGNOSIS AND SPINAL FLUID EXAMINATIONS

Cytodiagnosis is chiefly employed in the examination of cellular sediments of pleural, ascitic and cerebrospinal fluid.

The fluids which pathologically collect in the serous cavities are divided into two classes: (1) The transudates, which form as the result of some circulatory inadequacy, and (2) the exudates, which result from inflammatory processes.

Transudates have little or no fibrin and very few cellular elements and do not contain nucleo-albumin. Exudates contain nucleo-albumin and usually have a specific gravity above 1.018, while that of the transudates is lower than 1.018.

There are two simple methods for differentiating transudates and exudates. Moritz adds 2 drops of a 5% solution of acetic acid to the fluid to be tested. A heavy, cloud-like precipitate shows the fluid to be of inflammatory origin (an exudate). A transudate may produce a slight opalescence. Rivalta's test consists in dropping a drop of the fluid to be tested into a cylinder containing 2 drops of glacial acetic acid in 100 cc. distilled water. A nebulous cloud as the drop of fluid sinks shows an exudate.

For pleural fluids we should receive the material in centrifuge tubes about one-fourth filled with 2% sodium citrate salt solution. This prevents clotting. Having thrown down the sediment, the supernatant fluid is poured off, and in its place a 1% aqueous solution of formalin is added. After mixing and allowing to stand for about five minutes, centrifugalization is again repeated and, pouring off the supernatant formalin solution, we make smears from the sediment. This is either stained by a Romanowsky method or, after fixing with heat (burning alcohol), the smear is stained with haematoxylin and eosin. To attach sediment to slide add albumin.

With ascitic fluid it is usually sufficient to centrifuge the fluid, then decant off the supernatant fluid and drain by means of a piece of filter paper held at the mouth of the inverted tube. The sediment adheres to the bottom of the tube and is best emulsified with the small amount of fluid remaining by means of a bulb pipette. The material is sucked up, smeared out on a slide with a second slide as for blood and stained preferably with Giemsa after fixation. Haematoxylin-eosin staining brings out mitotic figures best. If the fluid has coagulated it is best to take a little of the coagulum and stain it with neutral red as for vital staining. It is difficult to dissociate the cells from the clot. I now make it a rule to collect a portion of the pleural or ascitic fluid in citrated salt solution in order to prevent coagulation. The material is then centrifuged and after removal of supernatant fluid with a bulb pipette the cell sediment is drawn up and smeared out on a slide for cell or bacterial

staining. By making smears as for blood beautiful preparations may be obtained. I prefer Giemsa for differentiating cells and Gram's staining for bacteria.

The wet Giemsa method described for blood gives good results with these sediments.

At the time of securing fluid for cytodiagnosis, cultures should be made on blood-serum for various pyogenic bacteria and, if tuberculosis is suspected, inoculation of a guinea pig is indicated.

Fluid from an empyema due to haemolytic streptococci is thin, dirty and serous, at times showing a bloody character, while that connected with pneumococcal empyema is frankly purulent. During the war it was found that immediate operation (excision of rib) with the haemolytic streptococcus fluid often resulted fatally, the better course being to aspirate from time to time and delay operation until the fluid became purulent. Of course with a pneumococcal empyema early operation is indicated.

The interpretation of cellular sediments is more difficult than many books would indicate, there being many factors which tend to complicate the findings.

The polymorphonuclears in purulent fluid often show fatty degeneration, swollen and faintly staining nucleus or a breaking up of the nucleus into small deeply staining masses (nuclear fragmentation). Such fragments in the smear may be confusing. The endothelial cells often show fatty degeneration in the cytoplasm and we often note bacteria and other cells which have been phagocytized by them. Where proliferation of endothelial cells is going on actively the cells show a rather deeply staining cytoplasm as compared with the light-staining cytoplasm of the cells in transudates. Some authorities attach importance to the Foulis' cells in connection with malignant processes in the peritoneum, particularly those associated with a malignant type of ovarian cyst. Such cells are large, often multinucleated and may show appearance as if budding.

The following differential points are important in cytodiagnosis:

1. A smear showing almost entirely lymphocytes with a few red cells and very rarely a polymorphonuclear indicates a tuberculous process.

2. Where a pyogenic process is engrafted on a tuberculous one, we have still the red cells, some degenerated lymphocytes, and in particular polymorphonuclears showing fragmentation of their nuclei.

3. When a hydrothorax results from chronic heart or kidney disease, the characteristic cell is the endothelial cell, which greatly resembles a large mononuclear. These cells often are arranged in plaques.

4. Some authorities consider that the cancer cells can be recognized by their occurring in masses and having a markedly vacuolated cytoplasm. It has been claimed that they contain glycogen by which fact we can distinguish them from endothelial cells which they so much resemble. If such cells should show mitosis the finding would be suggestive. For mitotic figures wet fixation with some bichloride fixative is best, followed by haematoxylin-eosin staining.

Jousset introduced *inoscopy* as a means of diagnosing tuberculosis. The fluid was allowed to coagulate and was then digested with an artificial gastric juice. The digested material was then centrifuged and the sediment examined for tubercle bacilli. This process does not seem to have met with much favor in this country. (Using sodium citrate obviates the necessity for digesting the coagulum.)

The same points will hold for ascitic fluid as for pleural fluid.

The *normal* cerebrospinal fluid is as clear as water. See p. 760 for other characteristics.

To withdraw spinal fluid for bacteriological examination or cytodiagnosis we use a sterile needle about 4 inches long for an adult, preparing the skin as described for blood cultures from a vein. Aspiration is responsible for many of the ill effects of lumbar puncture. The patient is placed on the left side with knees drawn up and head and shoulders carried forward to give the greatest possible space between the spinous processes by arching the spine. A line at the level of the iliac crests passes between the third and fourth lumbar vertebrae. Select a point midway between the spinous processes of these lumbar vertebrae and enter the needle two-fifths of an inch to the right of this point, pushing the needle inward and upward. Collect the material in two or three sterile test tubes, to avoid contamination of the entire sample by a drop of blood which may come out in the first portion. The presence of blood in very slight amount interferes with cytodiagnosis and globulin tests and, if present in more than a trace, it makes the colloidal gold test practically worthless. Make cultures on blood serum or blood agar followed by a cell count and then centrifugalize and examine the sediment as for pleural fluids. The supernatant fluid is reserved for spinal fluid Wassermann or Kahn tests, globulin tests and sugar reduction ones.

After the puncture the patient should drink a glass or so of water and remain in bed for a day, preferably with the head lower than the feet.

Cell Count.—A method of examination considered by neurologists as of differential diagnostic value is to count the number of cells in a cubic millimeter of the cerebrospinal fluid. The technique is to use a gentian-violet-tinged 3% solution of acetic acid. This is drawn up to the mark 0.5, and the cerebrospinal fluid is then sucked up to 11. After mixing, the cell count is made with the haemacytometer. Count all the cells appearing in the entire ruled area (9 large squares) and add one-sixth of this number to find the approximate total number of cells per cubic millimeter of spinal fluid examined as above. It is advisable to make the cell count of the fluid as soon after obtaining it as possible, the cells tending to degenerate or adhere to the glass of the tube. The latter tendency can be minimized by vigorous shaking before withdrawal of the fluid for counting. Normally we have only one to five cells per cubic millimeter, but in tabes or general paresis this may be increased to 50 or 100 cells or more (greatest at onset of disease).

It is customary to consider fluid containing blood as unsatisfactory for the cell count as well as for the globulin tests, but one can calculate the leukocytes due to blood content by counting the red cells and subtracting one leukocyte for each 750 red cells.

In general terms, excluding syphilis, it may be stated that:

1. A lymphocytosis indicates a tuberculous or poliomyelitis process. With these diseases, the fluid will probably be clear.

2. An abundance of polymorphonuclear and eosinophilic leukocytes indicates an infection with pyogenic organisms, in which cloudy fluid is the rule.

Meningism shows very few cells.

In lethargic encephalitis the spinal fluid is clear, colorless, pressure is normal, total protein is increased, there is a trace of globulin; the cells are variable, not over 100 per cubic millimeter, all mononuclears. Sugar is high and Lange is often in luetic zone.

Trypanosomiasis gives a cellular increase very similar to that of syphilis.

In the work of the French Sleeping Sickness Commission five cells per cubic millimeter was taken as normal.

Not only may trypanosomes be found in the spinal fluid, when they mark the setting in of the "sleeping sickness" stage of trypanosomiasis but a case has been reported of the presence of *Trichinella* embryos in the spinal fluid. A few cases have been reported of anthrax meningitis, in which anthrax bacilli have been found in the spinal fluid.

Miller gives the following table as to pleocytosis:

AVERAGE INCIDENCE OF LYMPHOCYTOSIS IN THE SPINAL FLUID
(Plaut, Rehm and Schottmuller)

Clinical diagnosis	Frequency	Remarks
Cerebrospinal lues.....	85–90%	Counts often over 100—may reach 1000 per c. mm.
Tabes dorsalis.....	90%	Counts usually under 100.
General paresis.....	98%	Counts average 30–60 cells per c. mm.
Secondary lues.....	30–40%	Moderate increase as a rule.
Multiple sclerosis.....	25%	Borderline counts.
Cerebral haemorrhage...	Frequency is variable	Cellular increase is apt to be a very moderate one.
Cerebral tumors.....		
Sinus thrombosis.....		

Demonstration of Tubercle Bacilli in the Spinal Fluid.—The following simple method, developed by Sheo-Nan Cheer, has been found very satisfactory.

Five cubic centimeters of spinal fluid is collected in a well cleaned centrifuge tube. To this, from one-third to one-half volume of 95% alcohol is slowly added, the tube being gently shaken all the while so as to obtain an evenly distributed cloud of coagulum rather than a single thick ring at the top. More or less alcohol may be added according to the density of the coagulum. In case very little or no coagulum is formed, a drop or two of dilute solution of egg albumin may be added. The optimal amount of coagulum is one that forms a milky appearance, such as one would call a "trace" in urine analysis.

The tube is then centrifuged at a fairly high rate of speed for one-half hour, or longer in case the precipitate is scanty. The supernatant fluid is then carefully decanted, leaving the albuminous precipitate in a very tiny drop of fluid. With a wire, or a capillary pipette, this is then transferred to three or four slides, care being taken not to touch the sides of the tube in withdrawing the loop, since the material may stick to the tube and be lost. One slide may be reserved for Gram stain or differential count, and the rest stained by the Ziehl-Neelsen method.

This method may also be used with urine. The solids should be thrown down first by low-speed centrifuging for five minutes and then the clear urine is treated as above.

Colloidal Gold Test (Lange's).—It is now generally accepted that this test is more diagnostic of general paresis than any other single test. The color changes in the first five tubes (1–10; 1–160) are so constant that the term "paretic curve" is applied to such findings. Of less diagnostic value are the so-called cerebrospinal lues curves where the color changes, though of less intensity than the paretic ones, are most marked in the third, fourth, fifth and sixth tubes (1–40 to 1–320). In various types of meningitis, other than luetic, the color changes are at times more marked in the tubes with the higher dilutions of spinal fluids (from 1–320 to 1–2560).

The paretic curve of the colloidal gold test generally runs parallel with a spinal fluid complement fixation or precipitation test and globulin increase. This agreement does not exist at all constantly for positive blood-serum complement fixation or precipitation tests and increased cell counts.

It may be stated that this test is of more importance in paresis than any single one of the four reactions of Nonne, viz. (*a*) blood serum, complement fixation or precipitation test (*b*) spinal fluid, complement fixation or precipitation test (*c*) globulin increase and (*d*) increased cell count of spinal fluid (pleocytosis). Of course, all of these tests should be carried out.

Preparation of the colloidal gold solution.—The method followed at the U. S. Naval Medical School is here described.

The following solutions made with triple distilled water are required:

1. 1.0% solution of gold chloride (Merck's).
2. N/5 solution of potassium carbonate.
3. 0.5% formaldehyde solution.

Procedure.—In a 2-liter Erlenmeyer flask, place 1000 cc. of triple-distilled water. (A large flask facilitates vigorous agitation at the end of the procedure.) Immerse flask in larger water bath. A suitable bath may be improvised by using an ordinary galvanized iron bucket on the bottom of which is placed a metal screen elevated 1 inch by turned-down sides. The water in the bath is brought to the level of the water in the Erlenmeyer flask as it rests upon the screen. Place a Bunsen burner under the bath and rapidly heat until the contents of the flask reach 60°C. Rotate flask occasionally during heating. Remove the flask and add from a burette 7.25% of an N/5 potassium carbonate solution. Rotate flask well and add while rotating 10 cc. of a 1% gold chloride solution delivered from a 10-cc. pipette. Rotate thoroughly and again place in water bath bringing contents of the flask to 92°–95°C. After reaching this temperature, remove from bath, rotate well and add while rotating 4.5 cc. of 0.5% formaldehyde solution. Continue to agitate vigorously by rotating flask until the solution passes through a deep amethyst to a red color. This usually requires from 5 to 8 minutes. The finished solution must be transparent and of a deep-red color when examined in transmitted light and by reflected light should have a slight brown shimmer, or metallic appearance. This is the final product, and it should conform to the following requirements:

1. Five cubic centimeters of the solution must be completely precipitated by 1.7 cc. of a 1% solution of sodium chloride in the time interval of one hour.
2. The solution must be neutral in reaction.
3. It must give a typical reaction with a known paretic cerebrospinal fluid.
4. It must produce no reaction greater than a No. 1 with known normal cerebrospinal fluid.

NOTE.—In the absence of a known paretic spinal fluid a simple method which has been found reliable is as follows: Set up a rack of ten tubes (using tubes as in Lange's test) and into each measure 5 cc. of colloidal gold and then the following amounts of N/5 sodium chloride.

TUBE NO.	AMT. OF N/5 NaCl
1	1.3 cc.
2	1.2 cc.
3	1.1 cc.
4	1.0 cc.
5	0.9 cc.
6	0.8 cc.
7	0.75 cc.
8	0.7 cc.
9	0.65 cc.
10	0.6 cc.

Under the above conditions the solution after standing six hours should be completely precipitated in not less than the first four tubes and not more than the first five; i.e.,

if only the first three tubes show complete precipitation the solution is "protected" and if more than five are precipitated the solution is too sensitive for the purpose for which it is intended.

N/5 sodium chloride solution can be prepared by adding 11.692 Gm. of sodium chloride (chemically pure) made up to a liter with triple distilled water.

If a solution results that is unsatisfactory in any of these respects, it should be discarded, and after a careful and thorough recleaning of all glassware, another solution made up. This procedure in our experience has always resulted in a satisfactory solution and takes less time and is less trouble than trying to "correct" a poor solution. Some specimens of gold chloride fail to give satisfactory solutions.

Gold chloride.—Merck's acid gold chloride is satisfactory. This reagent is made up in 1% solution with triple distilled water. Gold chloride usually comes in 1 Gm. bottles or ampoules and, as it is deliquescent, cannot be accurately weighed. It has, therefore, been found advisable to use the weight recorded on the container. The 1% solution should be well protected from light. If any other reagent than Merck's is used it may be necessary to modify the procedure given above.

N/5 potassium carbonate solution.—As all samples of potassium carbonate are not anhydrous, even though so labeled, it is necessary to have control over this factor also. A solution of a product of as high a purity as can be obtained is made up and adjusted against N/1 H_2SO_4 using methyl orange as indicator. This reagent should be adjusted before use.

Preparation of the 0.5% formaldehyde solution.—The most important step in the preparation of the colloidal gold solution is obtaining a satisfactory formaldehyde solution.

A 0.5% solution of formaldehyde made simply by diluting formalin to the desired strength is unsatisfactory, as the commercial product contains a large quantity of methyl alcohol and other impurities. In order to obtain a pure 0.5% formaldehyde solution, commercial formalin must be distilled, the distillate assayed to determine its concentration and then properly diluted according to the following technique:

Place 250 cc. of formalin in a 500 cc. distilling flask, distil and reject the first third of the total, collecting the second third, and allow the last third to remain undistilled. The portion saved will be free from methyl alcohol and other impurities; this is now assayed for strength and properly diluted to 0.5%.

The most satisfactory method of assay is one based on the method laid down in the U. S. Pharmacopoeia: Measure with a pipette 2 cc. of the distillate and place in a 150-cc. Erlenmeyer flask, add 10 cc. triple-distilled water and follow this with exactly 50 cc. of normal volumetric solution of potassium hydroxide. Place in a bath of boiling water and heat; now slowly add 50 cc. of U.S.P. solution of hydrogen dioxide which has been neutralized to litmus. Heat cautiously shaking occasionally for about ten or fifteen minutes. Remove from water bath and cool, then add 5 cc. of litmus indicator and titrate with normal volumetric sulphuric acid solution. The amount of N/1 H_2SO_4 required, subtracted from 50, and the result multiplied by 0.03002 gives the weight of formaldehyde in the 2 cc. taken for assay; this quantity multiplied by 50 gives the strength per centum of the redistilled formaldehyde solution. To calculate the amount of the strong formaldehyde solution required for making 100 cc. of 0.5% strength, divide 50 by the strength per centum.

Water.—All water used in the preparation of the stock solutions and the reagent itself must be triple distilled. A block tin condenser is used and rubber connections must be avoided. A very satisfactory condenser can be made from a piece of block tin tubing about 60 cm. long, bent in the form of an inverted "U" with one long arm and one short arm. The short arm is passed through a tinfoil-covered cork into the distilling flask and the long arm is surrounded by a glass or metal cooling jacket through which water circulates. The lower end of the tube passes into a flask placed to catch the distillate.

To the first distillate obtained is added a small quantity of sodium carbonate (about 1 Gm. per liter). We believe this to be very important, as it frees the water from all traces of ammonia that may be present due to absorption of the gases in the laboratory, and as a further precaution against impurities. Of the second distillate, the first portion collected, to the amount of 10% of the total volume, is rejected. 80% is collected and the last 10% allowed to remain in the flask. The collected second distillate is redistilled, discarding the first 10% and collecting about 80% which is the third distillate or the triple-distilled water to be used in preparing the colloidal solution and for washing all glassware.

Glassware.—New glassware should be used, or that which has not been used for any other purpose. It is first cleaned with hot soapy water and then rinsed with aqua regia. After this, all flasks, pipettes, etc., are completely filled with tap water many times to drive out all traces and fumes of aqua regia and then rinsed with distilled water, and just before use with triple-distilled water. Pipettes, burettes and volumetric flasks should be accurately calibrated.

Actual test.—Put 11 clean dry test tubes in a rack and deposit in the first tube 1.8 cc. of a 0.4% solution of sterile saline. Into the other 10 tubes put only 1 cc. of the 0.4% saline. Into the first tube deliver 0.2 cc. of spinal fluid and mixing thoroughly we have 2 cc. of a 1–10 dilution. Withdraw 1 cc. from the first tube and add to the 1 cc. of saline in the second tube. This gives 2 cc. of 1–20. Continue the process until the No. 1 to No. 10 tubes contain 1-cc. quantities of the various dilutions from 1–10 to 1–5120.

Tube 11 contains no spinal fluid but only 1 cc. of the saline and serves as a control.

To each of these 11 tubes add 5 cc. of the colloidal gold reagent. The color changes are usually read after the tubes have stood over night at room temperature.

The proper color of the control in tube 11 should be salmon red or old rose and the fluid should be perfectly transparent. When the color is changed in tubes containing dilutions of the spinal fluid we record one showing a bluish tint as 1. When the change is a lilac we record it as 2. A distinct blue is marked as 3 and a pale blue as 4. When decolorization is complete there is the highest color change, which is noted as 5.

NOTE.—An important consideration is that even minute traces of blood, accidentally present in a fluid from puncturing a small vessel, will give erroneous Lange readings. Even ¼ drop of blood in 10 cc. of fluid will give changes which are generally most marked in tubes 2 and 3.

Mastic Test.— The mastic test as devised by Cutting is apparently of assistance in the diagnosis of syphilis of the nervous system. Its great value lies in the ease of preparation from an effective gum mastic, and in the keeping qualities of the stock solution.

The stock mastic solution is made by dissolving 10 grams of pure gum mastic in 100 cc. of absolute alcohol; this is filtered and preserved in tightly stoppered bottles in which it will keep indefinitely. One part of this stock solution is added to nine parts of absolute alcohol and the mixture is then sprayed into forty parts of distilled water, producing an opalescent solution which keeps well for several days and is the solution used in the actual test. The absolute alcohol used in the test should be of highest purity and not merely dehydrated alcohol.

Make a 1.25% solution of sodium chloride (NaCl) in distilled water and to this add 1 cc. of a 0.5% solution of potassium carbonate for each 100 cc. of NaCl solution used.

Six test tubes are placed in a rack; in the first tube is placed 0.5 cc. of the spinal fluid, to be tested, and 1.5 cc. of the combined 1.25% NaCl and 0.5% potassium carbonate; place in tubes Nos. 2, 3, 4, 5 and 6, 1 cc. each of the 1.25% NaCl and 0.5% potassium carbonate solution. Remove with a 1-cc. bulb pipette, 1 cc. of the mixture from the tube No. 1 and place in No. 2 and mix; carry 1 cc. from tube 2 into tube 3, advancing until tube 5 is reached. The 1 cc. removed from this tube is discarded, tube 6 being the control and not receiving any of the spinal fluid mixture.

Mix the fluid in the test tubes by shaking or stirring with a clean glass rod. Add 1 cc. of the mastic emulsion to each of the six tubes and mix again thoroughly. Allow to stand in a warm place (room temperature) for from twelve to eighteen hours, or incubate at 37°C. for six to twelve hours and centrifuge before reading.

In positive cases the mastic will be precipitated in the first one, two, three, or four tubes (or higher in strongly positive cases) leaving the supernatant fluid clear and the mastic a white, flocculent precipitate at the bottom of the tube, though in some cases the opalescent appearance of the fluid remains the same, with, however, a fine white precipitate of mastic at the bottom of the tube. The control should never change its appearance.

Globulin Increase Tests.—The test generally used is *Noguchi's butyric acid* one. Deliver into a small test tube 0.5 cc. of a 10% solution of butyric acid in 0.9% salt solution. Then add 0.1 cc. of spinal fluid. Bring to a boil over a flame and add 0.1 cc. of N/1 NaOH solution. If there is a considerable increase of globulin a flocculent precipitate appears in a few minutes or at any rate in one or two hours. Fluids with a normal content or only slight increase show only a slight opacity.

The odor of the butyric acid is very objectionable and in our laboratory we use the *Ross-Jones* method. In this one deposits in a small tube about 1 cc. of satu-

rated solution of ammonium sulphate. On the surface of this column we deposit 1 cc. of spinal fluid. If globulin increase is present a turbid ring appears within a few seconds at the junction. Normally there is no sign of a ring. This test is a modification of Nonne's Phase I reaction.

A test that is not in general use is strongly recommended by Miller. It is known as *Pandy's test*. To carry it out prepare a saturated solution of carbolic acid crystals in distilled water. Place 1 cc. of this reagent in a small test tube and add 1 drop of spinal fluid. In a normal fluid only the faintest opalescence is observed, but in a fluid with globulin increase a smoke-like white cloud develops instantly where the drop comes in contact with the reagent. Miller gives the following table as showing the average frequency of the various reactions in syphilis of the central nervous system.

	Paresis	Tabes dorsalis	Cerebrospinal syphilis
Blood Wassermann	98–100%	70%	70–80%
Spinal fluid Wassermann	97%	60–80%	85–90%
Pleocytosis	98%	85–90%	85–90%
Positive globulin test	100%	90–95%	90–95%
Colloidal gold test	98–100%	85–90%	75–80%
	Paretic curves	Luetic type of curve	Luetic curve

For further comparison of the blood and spinal fluid Wassermann see page 284.

Poliomyelitis in addition to small mononuclear increase together with rather characteristic large mononuclear cells shows a globulin increase.

Chemistry of Cerebrospinal fluid (see p. 721).

Technique of Obtaining and Preparing Serum for Swift-Ellis Treatment.—One hour after the intravenous injection of the desired dose of arsphenamine or neo-arsphenamine withdraw, observing strict asepsis, 40 cc. of blood into a 100-cc. Erlenmeyer flask. After standing about two hours in the ice box gently rotate flask to free clot from side of the container and replace in the ice box. The following day 12 cc. of serum is pipetted off and diluted with 18 cc. of sterile normal salt solution. This 40% serum is then heated in a water bath at 56°C. for one hour. The serum is then ready for use.

Treatment of General Paresis by Inoculation of the Malarial Parasite.—There have appeared reports of the successful treatment of paresis by the injection of blood of persons with active malaria. Cases showing a benign tertian infection are preferably used since transmitting a malignant tertian infection would be attended by dangers overbalancing any probable benefit. About 1 or 2 cc. of the malarial blood is injected subcutaneously.

While a patient with general paresis is already infected with syphilis and therefore presumably resistant to further infection, yet it is possible that a new infection might follow inoculation of blood containing treponemata, perhaps with strains that would give rise to new and serious lesions. Therefore blood should be obtained from donors whose blood gives a negative Wassermann or Kahn reaction.

Of interest in this connection is the statement, sometimes made, that during the paroxysm, or at the time of active schizogony, the blood Wassermann is positive, becoming negative during the latent period. This observation, if confirmed, is of practical importance since a positive Wassermann due to the malaria may lead to rejection of a donor at the time when his blood carries the infection. From a study by us of the Wassermann reaction in 18 cases of benign tertian malaria, selected as prospective donors, the following conclusions were drawn: (1) In active benign tertian malaria, the Noguchi reaction is almost always negative; (2) in not over 50% of patients with malaria and a history of treated syphilis there will be a partial inhibition of haemolysis in the Noguchi test; (3) the Noguchi reaction becomes negative in these cases after quinine therapy.

Relapsing fever treatment.—While the majority of investigators in fever therapy of general paresis have employed benign tertain malaria others have used relapsing fever, recommending, however, the use of a spirochaete strain that produces an infection responding to arsphenamine.

Cerebrospinal Fluid in Differential Diagnosis

(After Fremont-Smith and Ayer in Journal of American Medical Association)

	Disease	Initial pressure: mm. of spinal fluid horizontal position	Rise on jugular compression	Appearance	Cells per cmm.	Globulin	Protein mgm/100 cc.	Sugar mgm/100 cc.
Normal	Lumbar Cisternal Ventricular	70–190	Prompt	Clear colorless no clot	0–5 0–5 0–3	0	15–45 10–25 5–15	50–75 50–75 55–80
	Blood plasma	——	——	——	——	——	6,200–8,200	70–110
	Meningismus	+	N	N	N	0	N or low	N or +
	Brain abscess	+	See brain tumor	Clear and colorless to turbid clot ±	+	±	Slight increase	N or +
	Acute purulent meningitis	+	N	Clear to purulent faint yellow ± clot	+	+	+	Low
	Acute anterior poliomyelitis	+	N	Slight opalescence rarely turbid faint yellow ± delicate fibrin web ±	+	Slight +	Slight +	N or slight increase
	Tuberculous meningitis	+	N	Opalescent to turbid faint yellow ± delicate fibrin web	+ Mononuclears	+	+	Low
	Encephalitis lethargica	N	N	N	N or slight increase no polys	±	N or slight increase	N or slight increase
	Acute syphilitic meningitis	+	N	Clear to turbid faint yellow ± fibrin clot	+	+	+	N or slightly low
	"Meningo-vascular syphilis"	±	N rarely delayed	N Rare fibrin clot	+	+	+	N
	Progressive parenchymatous syphilis	+	N	N Rare fibrin clot	+	+	+	N
	Late inactive forms	N	N	N	N	N	±	N

For additonal information as to chlorides, nonprotein nitrogen, etc., in the above diseases see next page (648).

CEREBROSPINAL FLUID IN DIFFERENTIAL DIAGNOSIS.—(*Continued*)

	Disease	Chlorides (as NaCl) mgm/100 cc.	Non-protein nitrogen mgm/100 cc.	Gold sol	Comment
Normal	Lumbar Cisternal Ventricular	720–750	12–18	0000000000	Sugar and chloride values apply to fasting nonfebrile individuals.
	Blood–plasma	570–620	18–30	————	
	Meningismus	N or low	N	N	Large amount of fluid with relatively slight drop in pressure.
	Brain abscess	N	N	Variable	Polys nearly always present. If complicated by septicemia or high fever chlorides may fall to low levels (brain abscess represents one form of "Aseptic Meningitis").
	Acute purulent meningitis	Low	N	Variable	Sugar may be but slightly decreased at outset. Often falls rapidly to under 10 mgm. Chlorides rarely below 640 unless septicemia is present. Meningococci and influenza bacilli found in smear and culture with difficulty; pneumococci, streptococci and staphylococci easily. Indol present in influenza meningitis.
	Acute anterior poliomyelitis	N or slight decrease	N	Variable	In Preparalytic Stage Polys may exceed 80 %—Rapid change to mononuclears. With gradual decrease in cells protein increases for two to three weeks.
	Tuberculous meningitis	Very low	N	Variable	Excess fluid. Excess polys very early and in infants. Chlorides usually below 640 mgm/100 cc. Progressive fall in sugar which may be high at outset, to under 30 mgm/100 cc. or lower. Tubercle bacilli found in clot or sediment, guinea pig inoculation positive.
	Encephalitis lethargica	N	N	Variable	Sugar is normal unless blood sugar is elevated. Over 50% of cases have normal cell count, which rarely exceeds 60 cells. Protein increase when present is slight—rarely reaching 100 mgm/100 cc.
	Acute syphilitic meningitis	Slightly low	N	Strong reaction zone variable	Wassermann reaction nearly always positive.
	"Meningo-vascular syphilis"	N	N	Variable	Wassermann reaction nearly always positive.
	Progressive parenchymatous syphilis	N	N	Paretic or luetic	Wassermann reaction always strongly positive. (Includes Tabes, Paresis, and Optic Atrophy).
	Late inactive forms	N	N	Weak luetic ±	Wassermann reaction weakly positive or negative.

NOTE.—Heavy type stresses most significant tests.

CHAPTER XXXVII

RABIES, SMALLPOX, VACCINIA AND THE FILTERABLE VIRUSES

Rabies.—Rabies is a disease of dogs and wolves, but is communicable to man and domesticated animals. The virus, whatever it may be, resides in the saliva and nervous structures. It is destroyed by a temperature of 50°C. In man the period of incubation is usually from three weeks to three months, but may be shorter or may extend over one year.

Bites about the face, hands and those with marked lacerations are particularly serious. Bites of rabid wolves give about four times as great a mortality as those of dogs. In the dog there are two types of the disease—dumb rabies and furious rabies.

Pasteur treatment.—By inoculating rabbits subdurally with an emulsion of the brain or spinal cord of a rabid animal, and successively the medulla of this rabbit subdurally into other rabbits, we finally so increase the virulence of the infection that rabbits die in six days. Beyond this it is impossible to increase the virulence of the virus which is then termed "fixed virus." The pathogenic power of this virus is also changed so that it is not apt to cause rabies if injected subcutaneously. To attenuate this virus the spinal cord of the rabbit is removed and is dried over caustic potash at a temperature of 23°C. The cord is divided into segments about 1 inch in length. Drying for about fifteen days seems entirely to destroy the virus.

To prepare the material for prophylactic injections a small portion of the cord is emulsified with normal salt solution and injected subcutaneously. The usual American method is to commence with a cord that has been desiccated only eight days. At first injections are given daily, and it is possible to inject three-day cords by the sixth day. The immunity is "active" and the immunizing agent is a "vaccine." Like vaccine virus the product can be preserved (for about a month) by the use of glycerin and cold storage so that it is now possible to send the material for inoculation from the laboratory preparing it. The treatment lasts for about twenty days.

Antirabic serum has been prepared by injecting sheep with emulsions of rabbits' cord and brain—at first intravenously, then subcutaneously but this serum is only of value in sensitizing vaccine.

Other methods of treatment are:

1. *The Harris method.*—In this the brain and cord are ground up while frozen by means of CO_2 snow and the frozen tissue dried over

H_2SO_4. The process of drying lasts about two days and the virulence of the virus is reduced one-half. The potency of the virus, when kept at 0°C., holds for six months at least.

2. *The Cumming method.*—In this the brain is emulsified in saline and dialyzed with weak formaldehyde solution. In this method the virus is so attenuated that injections do not produce rabies on intracranial inoculation.

3. *In the Hogyes method* the fresh virulent cord is injected but so diluted in strength that it acts as does an attenuated virus.

4. *Phenolized method.*—Fermi, and more recently Semple, have used virus which has been killed by the application of strong (1% or 2%) phenol, then further diluted to preservative strength (0.5%) for injection.

Diagnosis.—In the diagnosis of rabies in dogs it is preferable to preserve the animal alive so that the development of the symptoms may be observed.

FIG. 199.—Two nerve cells of hippocampus major (smear preparation) showing *Negri bodies*. *A*, *Negri bodies*; *B*, inner bodies within the *Negri bodies*. (*After Reichel, American Veterinary Review.*)

In case the dog has been killed, it may be possible to make a diagnosis by means of the Negri bodies. These are round or oval bodies from 1 to 20μ in diameter, which may be found in the nerve cells especially those of the cornu Ammonis (hippocampus major).

These bodies were first described by Negri in 1903. In street rabies large amoeboid forms from 18 to 23μ may be found, while in the nerve tissues of animals with "fixed" virus only minute forms, 0.5μ or less, may be detected. The fact that the virus will pass through a Berkefeld filter is no argument against its protozoal nature.

Calkins considers it to be of rhizopod affinity. The name *Neuroryctes hydrophobiae* has been given it. The bodies are present four to seven days before the onset of symptoms. They may be demonstrated by staining smears of gray brain substance by some Romanowsky method, especially by the Giemsa stain. The smears should be made by mashing the thin slice of the outer gray matter rich in ganglion cells taken from (1) cornu Ammonis, (2) region of fissure of Rolando—in dog, crucial sulcus—or (3) cerebellum, with a cover glass against the slide. Afterward the cover glass is gently drawn along the slide.

The smear on the slide is then fixed in methyl alcohol for two or three minutes, washed with water and covered with a stain made by adding 3 drops of sat. alc. sol. of basic fuchsin to 10 cc. of distilled water and then adding 2 cc. of Löffler's methylene blue solution. The stain on the slide is then steamed gently and afterward washed with water and dried.

As their relation to the nerve cells is more or less disturbed by such a method it is preferable to fix brain tissue from the region of the cornu Ammonis for five to seven hours in Zenker's fluid, then to imbed in paraffin and make sections. These are stained with Giemsa's stain and the Negri bodies are brought out as lilac-red bodies in the blue cytoplasm of the nerve cells. It is necessary to differentiate in 95% alcohol.

In the Lentz method the 3μ sections, after removal of the paraffin, are flooded with absolute alcohol. They are then stained with a 0.5% solution of eosin in 60% alcohol for one minute. Wash in water and next stain for one minute in Löffler's methylene blue. Again wash in water. Apply Lugol's solution to the section for one minute and then differentiate alternately in methyl alcohol and water until the section is pink. After washing in water, again stain with Löffler's blue for one-half minute, then wash in water and dry carefully with filter paper. Now differentiate in alkaline alcohol (1 drop of a 5% solution NaOH in 30 cc. absolute alcohol) until the section is pink, then quickly differentiate in acid alcohol (1 drop 50% acetic acid in 30 cc. absolute alcohol) until a slight blue outline to the ganglion cells is obtained. Treat rapidly with absolute alcohol and xylol and mount in balsam. The Negri bodies show as light carmine-pink bodies on the light-blue ground of the ganglion cells. In the interior of the pink bodies, dark-blue dots or rings may be observed.

This method can be used for brain smears also.

In addition to examining for the Negri bodies, a rabbit or guinea pig may be inoculated subdurally with a sterile salt-solution emulsion of the medulla of the dead dog. Inoculation is of value in controlling the microscopical diagnosis of rabies, and also in replacing it in cases where there is too much decomposition to prevent satisfactory smears being made. In the latter cases occasionally treatment with glycerin may kill off the contaminating organism so that inoculations are possible.

If the brain and medulla of the dog are to be sent to a laboratory for examination they should be packed in ice or placed in equal parts of glycerin and water. Sterilize the diluted glycerin by boiling, allow to cool, and drop the pieces of brain tissue into this. This does not kill the virus.

When from advanced putrefaction, or other causes, the Negri bodies cannot be found the changes in the Gasserian ganglia may give a diagnosis. In typical lesions the ganglion cells are more or less completely destroyed and replaced by cells of other types.

Immediate treatment.—When a person is bitten by a dog suspected of being rabid the following simple measures should be instituted. The dog should be kept under observation in a safe quiet place and, in case rabies exists, will show clinical evidence of rabies within five days and will die shortly afterward. When the animal dies the head and several inches of the neck should be removed and sent to the nearest laboratory.

The thorough cauterization of the dog-bite wound with pure *nitric acid* (no other cautery is efficient), as soon as possible after the bite, is imperative even when the Pasteur treatment can be given later.

Smallpox.—The etiology of this disease is very obscure, the virus being grouped under the Chlamydozoa. Smallpox and vaccinia are often classed as filterable viruses. Park, however, was unable to pass the virus of vaccinia through a Berkefeld filter with 40 pounds pressure, though his failure may have been due to lack of sufficient dilution. He did find that the virus would pass through the finest filter paper.

In 1892 Guarnieri noted cell inclusions in the cornea of rabbits inoculated with smallpox and under the name *Cytorhyctes variolae*, Councilman has described what he regards as a protozoon invading cell nuclei. Probably the inclusions represent cellular reactions rather than the causative agent itself. Certainly ordinary bacteria are not concerned in the etiology of smallpox. The virus is not only contained in the skin lesions but also in nasal and buccal secretions, the disease being communicable before the eruption appears.

The period of incubation of variola is usually about twelve days. Leake states the most important diagnostic points in the order of their importance, are the distribution of the eruption, the course of the disease, the individual lesions, and inoculation tests.

Paul's test.—This is an inoculation test and according to Leake it is the most useful laboratory procedure in the diagnosis of smallpox. He also states it consumes valuable time, and furthermore has an element of uncertainty on the dangerous side; that is, the atypical cases of smallpox (atypical by reason of the stage at which they are seen, or by reason of their modified character) are likely to give negative Paul reactions, causing a dangerous implication of security.

The test is carried out as follows: The contents of a vesicle or pustule is inoculated into a rabbit's cornea. 40 to 72 hours after inoculation the eyeball is enucleated, fixed in strong sublimated alcohol and examined for the characteristic whitish papules and the microscopic Guarnieri bodies in the corneal tissue.

Monkeys are susceptible to both smallpox and vaccinia.

Inoculated cutaneously, the rabbit shows a somewhat typical eruption with cowpox material, but does not show characteristic lesions with smallpox material.

Many believe that vaccinia is simply smallpox permanently modified by animal passage and it is stated that repeated passage of smallpox virus through calves produces vaccine virus. Inoculation of calves with smallpox virus is a most uncertain procedure, however, and Park states that he has been unable to obtain success after many such experiments.

Vaccination carried out in the proper manner with a potent vaccine will result in a reaction which may be classified under one of the following forms:

The *primary reaction or vaccinia*, indicating absence of immunity to smallpox prior to this vaccination, in which the zone of redness, rather narrow from the third to the seventh day, begins a sudden spread about seven days after vaccination and reaches its broadest diameter in eight to fourteen days after vaccination, rapidly disintegrating and disappearing thereafter. The reaction is accompanied by vesicle formation.

The *accelerated or vaccinoid reaction*, which indicates partial immunity in which the broadest redness is reached and passed in three to seven days after vaccination. Vesicle formation occurs.

The *immediate reaction or reaction of immunity*, which indicates full protection against smallpox, in which the broadest redness is reached and passed in eight to seventy-two hours after vaccination. The redness is accompanied by a slight elevation of the skin, which can be felt by passing the finger lightly over the vaccinated area. Vesicle formation usually absent.

As to confusing a reaction of immunity with an ordinary protein reaction, Leake states the protein reactions as shown by the usual skin tests (not subcutaneous) have an altogether different time relation from that of the reaction of immunity to smallpox. The former are rapid, appearing and reaching their maximum within about one-half hour, while the reaction of immunity to smallpox reaches its maximum in not less than eight hours after vaccination, and usually in more than twenty-four hours after vaccination. The protein reaction has faded before the reaction of immunity has begun to appear.

Vaccination.—Leake gives the multiple pressure or prick method (acupuncture) as probably the best method of vaccination. This consists of a shallow, tangential pricking of the cleansed, but not irritated, skin with a needle, through a drop of smallpox vaccine, covering an area not greater than one-eighth of an inch (3 millimeters) in diameter. This gives little chance of accidental infection and the eruption is typical. Acetone has been found satisfactory for cleansing the skin. It is somewhat more efficacious and rapidly drying than alcohol. The needle, which should be new, sharp, and sterile, is not thrust into the skin, but is held quite parallel or tangential to it, with the forefinger and middle finger of the right hand above

needle and the thumb below, the needle pointing to the operator's left. The needle should be crosswise of the arm so that the thumb of the operator is not impeded by hitting the skin. The side of the needle point is then pressed firmly and rapidly into the drop about thirty times within five seconds, the needle being lifted clear of the skin each time. The rapid to and fro motion of lifting the needle and pressing it against the skin should be quite perpendicular to the skin and needle, and not in the direction of the needle. In this way the elasticity of the skin will pull a fraction of an inch of the epidermis over the point of the needle at each pressure so that the vaccine is carried into the deeper epithelium (cuboidal prickle-cell layer), where multiplication takes place most easily. If the skin has not been unduly rubbed in cleansing, and if the motion is entirely perpendicular to the needle, no signs of bleeding will occur and all evidence of the punctures will fade out in less than six hours. Immediately after the punctures have been made the remaining vaccine is wiped off the skin with sterile gauze and the sleeve pulled down, the whole operation of puncturing and wiping taking less than ten seconds. With strong vaccine a single pressure not infrequently gives a "take." Only six pricks or punctures were formerly advocated; comparative tests showed this to be inferior to the scratch method in percentage of "takes." By the use of thirty pricks this difficulty has been overcome, and the percentage of "takes" is as high as with any other safe method. For primary vaccinations, where the mildest possible "take" is desired, and where other attempts with highly potent vaccine will be made promptly if the first is unsuccessful, the number of pricks may be reduced to ten, or even to a single prick.

The disadvantages of this method, which it shares with some other methods, are, first, that without demonstration and practice the technique of applying the proper pressure may not easily be acquired, and second, that without due care an area larger than one-eighth of an inch (3 millimeters) in diameter may be covered by the insertion. In regard to the first point, the difficulty is usually that the needle is not pressed in the right direction or that the pressure is not firm enough. Provided the needle is held quite tangential to the curve of the arm, and the direction of motion is quite perpendicular to the needle, it is difficult to make the rapid pressures too firmly. In regard to the second point, motion from the wrist with the arm held rigid is usually more accurate than whole-arm motion.

The advantages of this method are its mildness and painlessness, the fact that it is more rapid than any other effectual and safe method, the fact that no control site is necessary, since the evidence of trauma due to the operation has disappeared before the first observation for an early reaction is made, and the fact that the vaccine is wiped off immediately, so that the uselessness of a dressing is obvious to the person vaccinated.

The important points in vaccination are (1) a small superficial insertion (not over $\frac{1}{8}$ in. in any diameter), (2) keep the arm cool and dry and (3) keep the vaccine below freezing if possible.

Vaccinia.—Vaccinia is a disease produced artificially by the injection of vaccine virus obtained from the calf. The material for vaccine is taken from vesicles not more than one week after the inoculation. The most potent material is in the pulp at the base of the vesicle and

not in the lymph which exudes from the vesicle. The pulp is ground up and mixed with an equal amount of glycerin, which acts not only as a preservative but as a mild antiseptic for nonsporing bacteria. The calves are autopsied after the pulp has been curetted from the inoculated skin of the abdomen to be sure that no disease exists in them. The virus is afterward tested for pus organisms, tetanus, and foot-and-mouth disease.

Very important is the test for tetanus. Cultures are grown anaerobically and aerobically for at least seven days. One to two days after appearance of anaerobic growth and also seven days later inoculations into mice are made and the mice are observed for seven days to note evidence of tetanus. If found free from any harmful germs the vaccine is then tested upon rabbits for potency.

Guarnieri in 1892 first observed small bodies near the nucleus of infected epithelial cells. He called them *Cytorhyctes vaccinae*. Calkins regards these bodies as well as the Negri bodies as being rhizopods and the distributed chromatin as idiochromidia (granules of nuclear chromatin within the cytoplasm).

The Filterable Viruses

The first disease of which the virus was found to be capable of passing through the finest porcelain filter was that of foot-and-mouth disease (Löffler and Frosch, 1898).

The filter which is ordinarily used for testing for the passage of disease agents is the Berkefeld filter, one made of diatomaceous earth. The filter should be new and sterilized before use. The material should be diluted with saline before filtering. One may use slight suction from a filter pump. The filtration should occupy only a short time, not exceeding two hours.

The term filterable virus is applied to a virus so minute that it passes through the pores of a porcelain filter that is capable of keeping back bacterial organisms as small as *B. melitensis*.

The characteristic of filterability should be attributed to any microorganism with reserve, inasmuch as filterability depends on a number of conditions not all of which may be recognized and some of which at least are probably not subject to experimental control.

One of such conditions has to do with filters, all varieties of which exhibit great variability in the size of their pores, and in the properties of adhesion and adsorption. Another set of conditions relates to the suspending medium—its pH, its salt ratios, and the presence of protective colloids. Further, there may be sundry determining states present within the organism itself, which, for example, may have ultramicroscopic phases and which may exhibit irregularly changes in size, vis-

cosity, electric charge, or other characteristics such as to permit or prevent passage through. Indeed, in respect to the characteristic of filterability as with other characteristics, it must be borne in mind that the microorganism probably is potentially capable of responding to a changing environment with almost infinite variety of morphologic or biologic alterations.

Consequently, the term "filterable" should be understood as stating that the organism in question was filterable at the time of and under the conditions of the particular experiment specified.

Of human infections due to a filterable virus, we have the following: Foot-and-mouth disease, trachoma, molluscum contagiosum, vaccinia, variola, rabies, typhus fever, measles, dengue, pappataci fever, poliomyelitis and coryza.

Hog Cholera Virus.—Very interesting is the history of the etiology of hog cholera or swine fever. This disease was supposed to be due to an organism of the hog cholera group, *B. aertrycke* (identical with *B. cholerae suum* and *B. suipestifer*). This organism belongs to the "enteritidis" group and is more common as a cause of food poisoning in man than the better known Gaertner bacillus. It is now known that the cause of this most important fatal disease of swine is a filterable virus.

This virus shows remarkable powers of resistance to external influences; thus it can be kept for months in animal tissues. It is not destroyed by drying and withstands a temperature of 58°C. for two hours but not one of 72°C. for one hour. Cell inclusions have been found in smears from the conjunctivae of hogs sick with the disease. See Chlamydozoa. A very valuable prophylactic but not curative serum is found in the serum of animals recovering from the disease or in those immunized.

There are many other diseases of this nature which are important among the domesticated animals, such as pleuropneumonia of cattle, and African horse sickness. The viruses of pleuropneumonia of cattle and poliomyelitis have been obtained in artificial cultures. Some of these viruses seem related to bacterial infections and others to protozoal ones. These viruses differ as to method of transmission, pleuropneumonia of cattle being transmitted by inhalation, rabies and vaccinia by the cutaneous atrium, hog cholera by ingestion and many of these supposed to have protozoal affinities, as pappataci fever and horse sickness, by mosquitoes or midges.

As a rule these viruses are destroyed by a temperature of 55°C. in a few minutes.

CHAPTER XXXVIII

DISEASES OF UNKNOWN OR NOT DEFINITELY DETERMINED ETIOLOGY

Of Temperate Climates

Acute Articular Rheumatism.—Various bacteria have been reported as cause. The organism which seems the most probable cause is the short-chain coccus, *Micrococcus rheumaticus* of Triboulet and others. Inoculations of this streptococcus cause polyarthritis and pericarditis. Poynton and Paine have cultivated the organism from the cerebrospinal fluid in three cases of chorea.

The Common Cold.—Of all diseases common in man this condition has been surrounded by greater etiological and epidemiological obscurity than any other.

We are inclined to believe that the common cold (coryza) sets in when our resistance is lowered by alimentary tract disturbances, from exposure to variations in temperature, or following refrigeration and fatigue. Of course, many have held that the common cold was "catching" but the evidence offered in support of such a view has been academic.

Many bacterial organisms have been suggested as causative such as *B. coryzae segmentosus*, haemolytic and viridans types of streptococci, *M. catarrhalis*, etc. In 1914 Kruse brought forward evidence to prove that the etiological factor in coryza was a filterable virus.

Foster conducted experiments in which, by using the nasal discharge from typical coryza cases, diluting it with 10 or 15 times its volume of saline, then passing through a small Berkefeld filter and instilling 3 to 6 drops of the filtrate into the nasal cavity of 10 well men, he produced typical coryza in nine of these men in from eight to thirty hours. Cultures were made from the filtrate following Noguchi's spirochaete culturing method. The culture medium surrounding the piece of sterile tissue showed turbidity in from forty-eight to seventy-two hours and dark-field examination showed myriads of extremely active bodies which were thought to possess true motility rather than Brownian movement.

Filtrates from these cultures were instilled into the nasal cavity of 11 men and after a period of incubation of from eight to forty-eight hours all came down with coryza.

Epidemic Poliomyelitis.—Material from the cord of a child with the disease, when injected subdurally, intravascularly, or into the peritoneal cavity of monkeys, produced the disease in the animals inoculated. The virus has been passed through three generations of monkeys (Flexner).

The virus has been found in the brain, spinal cord, mesenteric and salivary glands of monkeys and may remain in the nasal mucosa of monkeys as long as five months. This observation makes probable the existence of human chronic carriers. With the possible exception of the rabbit only man and the monkey are susceptible. This would indicate that the virus is directly transferred from man to man. The virus is highly resistant to drying and light. It will remain alive for months in dust. It is not sterilized by pure glycerin during many months of contact. It is possibly transmitted by a biting fly, *Stomoxys calcitrans*. Opposed to this hypothesis is the fact that the virus has not been found in the blood.

Flexner and Noguchi have cultivated the virus of poliomyelitis by employing ascitic fluid to which had been added a fragment of sterile rabbit kidney and nutrient agar, this culture medium being covered with a layer of paraffin oil. The growth is obtained under anaerobic conditions. The minute colonies are composed of globular or globoid bodies from 0.15 to 0.3 micron in diameter. These bodies may be single or in chains or in masses. In older cultures bizarre forms are obtained. Monkeys have been inoculated with the cultures.

Rosenow considers streptococci having an affinity for the central nervous system as the excitants of the disease.

Foot-and-mouth Disease.—Due to an ultramicroscopic organism.

This is a highly contagious disease of cattle characterised by the appearance of vesicles in the mouth and about the feet. Rarely man contracts the infection through drinking the milk of infected animals. This disease is of great interest as having been the first of the filterable virus diseases to have been discovered (Löffler and Frosch in 1898).

Influenza of the Epidemic Type.—In the discussion of the *Bacillus influenzae* (Pfeiffer), p. 157, it was noted that much uncertainty existed as to the precise relationship of the organism to the disease for which it is named. For this reason, it was suggested that a better name for the organism would be "Pfeiffer's bacillus."

The work of Nicolle and Lebailly in which they reported a filterable virus as the cause of influenza and the work of Olitsky and Gates who found a filter passing organism to which the name *Bacterium pneumosintes* has been given have likewise been discussed on page 159.

Lethargic Encephalitis.—Following the recent pandemic of influenza there have been observed in Europe and America many cases of a febrile disease in which a stuporous or lethargic condition is prominent, together with frequent paralysis of the third nerve and less often of other cranial nerves.

It has certain resemblances to poliomyelitis but intracerebral injections of emulsions of brain of cases into monkeys fail to infect, thus negativing a similar cause. Loewe and Strauss claim to have cultured an organism similar to that of poliomyelitis. Some autopsies have shown microscopical areas of haemorrhage in various parts of the brain. About one-third of the cases die. The fact that such cases have been observed in former influenza pandemics suggests that the disease has a relation to influenza.

Measles.—Cause entirely unknown. Hektoen has shown that blood contains the virus.

Anderson has found that the virus of measles can pass through a Berkefeld filter and loses its infectivity after heating for fifteen minutes at 55°C. In infecting monkeys it was found that the blood of patients with measles was infective only just before and for about twenty-four hours after the appearance of the eruption. Mixed nasal and buccal secretions were infective for monkeys for about forty-eight hours from the time of the eruption. The scales from desquamating cases were not capable of infecting monkeys; hence it was thought that measles was not contagious during the period of desquamation. In the discussion on page 93 reference was made to the work of Tunnicliff, Ferry and Fisher in which they believe that measles is caused by a green-producing streptococcus.

Mumps.—Herb has implicated a diplococcus. Inoculations into Stenson's duct of monkeys successful.

Wollstein found a filterable virus present in the saliva of patients during the first three days of mumps. It was present at times up to the sixth day but never after the ninth day of the disease. This bacterial-free filtrate produced a disease similar to mumps when injected into the parotid glands and testicles of cats.

Rabies.—Probably the Negri bodies.

Roetheln (German Measles).—Nothing known.

Smallpox and Vaccinia.—Guarnieri and Councilman have implicated epithelial protozoa.

Spotted Fever of the Rocky Mountains.—Supposed to be due to a rickettsia-like organism *Dermacentroxenus rickettsi* transmitted by a tick, *Dermacentor andersoni.*

This disease is especially prevalent in the Bitter Root Valley of Montana and to some extent in the mountains of Idaho. It is an acute febrile affection with a tend-

ency to stupor. The eruption, which appears about the third to fifth day, is not unlike that of typhus fever and tends to become haemorrhagic. Gangrene of penis or scrotum may appear. It is transmitted by a tick, *Dermacentor andersoni*, which lives on domesticated and wild animals of the region. Destruction, by dipping, of ticks which attach themselves to sheep has been proposed as a measure for eradication of the disease. Ricketts found that the reservoir for the virus is to be found in ground squirrels, chipmunks, mountain rats, etc., and that ticks feeding upon them become infected and transfer the disease to man. Guinea pigs are susceptible as is also the monkey. Ricketts noted certain chromatin-staining bacteria in man and in eggs of infected ticks as possibly playing a part in etiology. Quite recently Wolbach has reported the finding in infected guinea pigs of organisms, possibly bacterial, showing granular and lanceolate forms, *Dermacentroxenus rickettsi*. They are particularly abundant in the endothelial cells of blood vessels. They were also found in the tissues of infected ticks after feeding. They are from 0.5 to 1 micron long by about 0.3 micron broad. Ricketts stated that his organisms were about the size of *B. influenzae* and showed as two lanceolate-shaped bodies. Wilson and Chowning, in 1922, reported the finding of piroplasm-like organisms in the blood of the disease. Ricketts proved that the virus was not filterable.

Spencer and Parker have shown that guinea pigs and monkeys may be successfully vaccinated against Rocky Mountain spotted fever by injections of phenolized emulsions of tick virus and suggest that the vaccine will confer immunity upon man.

Trachoma.—This form of granular conjunctivitis is supposed to be due to chlamydozoa or inclusion bodies and is classed as one of the filterable viruses.

The relation of the trachoma bodies to the Koch-Weeks bacillus is discussed under that organism. Noguchi reports the isolation of a Gram-negative bacillus with which he produces trachoma-like lesions in monkeys. It has even been suggested that trachoma may perhaps be another example of disease resulting from dietary deficiency.

Trench Foot.—This is a condition caused by defective circulation in the feet. It is brought about by prolonged standing in wet trenches during cold although not necessarily freezing weather.

The feet are swollen and usually show a reddish congestion although they may be white. There is a resemblance to frost bite and Raynaud's disease. The feet may show a gangrenous appearance. There was an idea that the condition might be due to a fungus infection but this view is no longer held.

Trench Nephritis.—Nephritis, showing oedema, increased blood pressure, headaches and lumbar pain, has been rather common in those exposed to the wet and cold of the trenches. It is relatively mild in type and the prognosis is good.

There has been a view that it might be connected with a spirochaete infection but such etiology is not generally accepted.

Typhus Fever.—It has been suggested that the cause may be a protozoon transmitted by vermin.

Work by Anderson and Ricketts has shown that the blood of human cases is infective for monkeys. The virus does not seem to pass through a Berkefeld filter and the epidemiology points to the body louse as the transmitting agent. Nicolle reported the filterability of the virus.

Hort states that blood recently taken from typhus patients will cause the disease in monkeys while the same blood which has been incubated several hours or days fails to produce the disease. Others, as well as Hort, doubt the etiological relation of the organism of Plotz to typhus fever or the mild form of the disease as seen in New York City and there known as Brill's disease. Tabardillo or Mexican typhus is the same as typhus.

The view that typhus is caused by certain minute organisms, possibly bacterial but, in view of arthropod transmission, probably protozoal, is gaining in support. This genus has been named *Rickettsia* and the type species, *R. prowazeki*. These were regarded by Ricketts, in his work on spotted fever of the Rocky Mountains, as chromatin-staining bacteria. These organisms ($3\mu \times .3$ to $.5\mu$) have been found in masses in infected lice, staining rather purplish with Giemsa. Similar organisms are considered as having a relation to trench fever (*R. quintana*).

In the diagnosis of typhus fever we attach great importance to an agglutination reaction (Weil-Felix reaction) which the serum of typhus patients has upon certain organisms of the *Proteus* group (X_2 and X_{19}). See page 185.

It seems probable that this is not a specific reaction as these *X* bacilli are neither causative organisms nor secondary invaders, but it is simply a heterologous one. The reaction appears during the first week of the disease but becomes quite marked in the second week and during convalescence. Thus a titre of 1 to 25 on the fifth day usually rises to 1 to 200 or higher by the end of the second week. The test is made either with living or dead cultures and is carried out as for typhoid agglutinations, preferably by the macroscopic method.

Varicella.—Cause is entirely unknown.

Whooping-cough.—Bacilli resembling the Pfeiffer bacillus have been implicated. Bordet-Gengou bacillus.

Of Tropical Climates

Ainhum.—A disease characterized by a constricting fibrous ring, especially of little toe, often leading to spontaneous amputation.

Blackwater Fever.—Considered as a malarial disease, but thought by some to be possibly caused by a protozoon—a *Piroplasma* (*Babesia*).

A disease usually occurring in patients with a malarial history and characterized by rapid febrile onset, early jaundice, asthenia, pain in loins and the pathognomonic haemoglobinuria.

Dengue.—Supposed to be due to a filterable virus transmitted by the *Aedes aegypti* and possibly other mosquitoes. It would now seem that *Culex quinquefasciatus*, formerly considered the transmitter, has been rendered improbable as a vector by recent experiments. A disease characterized by sudden onset, high fever for three or four days, and pains in the postorbital regions, back and about joints.

A remission occurs on the third to fifth day followed by a secondary rise of temperature and a measles-like eruption. Leukopenia and reduction in the percentage of polymorphonuclears. Virus exists in the blood and is filterable. See page 655.

Goundou.—Symmetrical bony tumors of nasal processes of superior maxillary bones.

Oroya Fever.—A disease with a fever characterized by profound involvement of the bone marrow producing very rapidly a pernicious type of anaemia. Pains of bones and joints marked. See Verruga.

The disease is chiefly found in towns situated in narrow, wind-protected valleys of the west side of the Andes, at elevations of from 3000 to 9000 feet. Townsend has suggested that a species of *Phlebotomus*, which is very prevalent, may be the transmitting agent. Experimentally the disease has been transmitted to monkeys by the bite of *D. andersoni*.

Barton isolated a paratyphoid bacillus from the blood of a patient, besides which other bacteria also have been isolated. In 1909, Barton noted certain rod-like organisms in the red cells of Oroya fever patients which he considered protozoal in nature.

Strong and his colleagues found in the blood of Oroya fever cases rod-shaped forms in the red cells, varying from 1 to 2 microns in length the red cells containing from 1 to 30 of these elements.

Intravenous inoculation of blood containing these elements into monkeys and rabbits was negative in result.

The organism formerly thought to be a piroplasm is now regarded as a *Bacterium*. The Harvard Commission proposed the name *Bartonella bacilliformis*.

Noguchi and his co-workers have isolated the organism in pure culture from the blood of a case of Oroya fever. These cultures when injected into monkeys intravenously reproduced Oroya fever, but when injected subcutaneously produced the lesions of verruga. This confirms the former opinion of the identity of the two

diseases. Carrion inoculated himself with the blood of a verruga lesion and died with Oroya fever (Carrion's disease). Noguchi considers the organism, *Bartonella bacilliformis*, as bacterial in nature. They are readily cultivated on horse blood agar and leptospira medium, and the finding of this organism in cultures from the blood, is the best method of diagnosis, as the organisms in the red cells of a case of Oroya fever are often difficult to find.

Rat-bite Fever.—This disease also known as sodoku is caused by the bite of rats. Rather common in Japan. Five weeks after bite when wound has healed, high fever sets in, cicatrix becomes inflamed with lymphangitis and swollen glands. The fever falls in a few days to be succeeded by other febrile paroxysms. An erythematous eruption accompanies the second paroxysm.

Supposed by Ogata to be due to a protozoon, and work by Schottmüller, in 1914, indicated that the cause is a *Streptothrix, S. muris ratti*. This finding was corroborated by Blake. The organism first invades the lymphatic structures and then the blood, giving a septicaemia. Various organs are later involved. Blake's case developed a powerful agglutinin for the specific *Streptothrix*. We have now come to accept a spirochaete, *Leptospira* (*spirillum?*) *morsus-muris*, which has spirals much like *Treponema*, as the cause.

Sprue.—A form of chronic diarrhoea characterized by diaphanous thinning of gut and ulcerations of buccal cavity.

Kohlbrugge found organisms resembling *Oidium albicans* in the intestines, oesophagus and tongue of cases of sprue. Beneke found bacilli in the tongue, oesophagus and intestines and considered these as causative, regarding the thrush-like membranous deposit as connected with the cachectic state and not causative.

Ashford states that he has found a species of *Monilia*, different from that of thrush, almost constantly in tongue scrapings and stools of sprue cases and he regards this species as the cause of sprue. He states that this organism is common in Porto Rico bread and thinks it possible that the disease is transmitted in this way. Wood has expressed the view that sprue is not infrequently mistaken for pellagra in the southern United States. Many now consider it a food deficiency disease and others a hepatitis or pancreatitis.

Trench Fever.—A group of imperfectly classified and ill-understood conditions has been noted in soldiers occupying trenches on the battle line under the designation "trench fever." The onset is quite abrupt, with severe frontal and postorbital headache, followed by rachialgia and pains in the extremities.

The fever rapidly rises to 102°–104°F. and falls rather abruptly to normal about the fourth day. A second rise is frequently noted, so that many of the temperature charts have a saddle-back character.

There is no rash and the disease is often diagnosed as influenza, although there are no catarrhal manifestations to justify such diagnosis. The spleen is frequently palpable. The pulse is often slow, the disease in this respect resembling the group of dengue-like fevers.

Besides the influenza-like type of trench fever another type has been described as the long-period one. In this there are frequent relapses, as many as five or six. The fever rise at such times is very short, only lasting a few hours or a day or so. A characteristic pain noted in trench fever is a cutaneous hyperaesthesia over the shins. The virus may be regarded as filterable in certain of its phases, as in the body louse, in which it apparently undergoes a cyclical development. The louse transmits the disease by its bite (inoculative). The cause is believed to be the *Rickettsia quintana.*

Tsutsugamushi.—A disease of Japan somewhat resembling typhus fever. Supposed to be due to a protozoon transmitted by the kedani mite.

Verruga Peruviana.—A disease of Peru formerly considered as a late stage of Oroya fever, a view recently reasserted by Noguchi.

The eruption of verruga somewhat resembles that of yaws and it was at one time suggested that verruga was simply yaws as influenced by high altitude. Strong and his colleagues found that they could infect rabbits intratesticularly and that lesions resembling those of man could be produced in dogs and monkeys by cutaneous and subcutaneous inoculations. The virus has been transmitted from monkey to monkey. The Wassermann reaction was negative. In extracts from the granulomatous lesions they found a very active haemolysin.

From the fact that it is possible to inoculate a person by rubbing verruga material on a scarified surface it would seem that the infection might be transmitted by insects—a *Phlebotomus*, according to Townsend.

Yellow Fever.—Due to a filterable virus transmitted by the *Aedes aegypti.* A disease characterized by sudden onset, rachialgia, albuminuria, jaundice and often haemorrhages about the third day. Pulse becomes slow even with rising temperature. Black vomit often precedes fatal termination. Virus exists in the blood and is filterable.

The virus is present in the blood of the peripheral circulation only during the first three days of the disease. A female *Aedes aegypti* sucking the human blood during the first three days from onset of fever may become infected but cannot transmit yellow fever to a second person until after the expiration of at least twelve days, during which time some development of the virus, of the character of which we are in ignorance, goes on in the mosquito.

Noguchi has reported a spirochaete, *Leptospira icteroides*, as cause. It can be cultured from the blood. See page 441.

Aitken and others in connection with an outbreak of very fatal yellow fever in Lagos (1925) reported that they obtained no evidence of the presence of leptospiral organisms, the investigation covering Pfeiffer reactions, cultivation experiments and inoculation of guinea pigs.

In connection with an epidemic of yellow fever at Parahyba, Brazil (1926), Sellards obtained negative Pfeiffer reactions with serum of eleven yellow fever convalescents, both with *Leptospira icteroides* and *Leptospira icterohaemorrhagiae*. As controls the serum of two guinea pigs immune to *Leptospira icterohaemorrhagiae* gave positive Pfeiffer reactions with *Leptospira icterohaemorrhagiae* and *Leptospira icteroides*. In Sellard's opinion these results favor the view of the identity of the two spirochaetes.

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A—PREPARATION OF TISSUES FOR EXAMINATION IN MICROSCOPIC SECTIONS

The *most important step in the preparation of sections of tissues for histological examination is proper and immediate fixation.* This step in the technique is often in the hands of the surgeon at the time of the operation or the physician at autopsy and it should be understood by them that a satisfactory diagnosis can be made only when the pieces of tissue are *at once dropped into a fixative.* Various protozoa, as amoebae, disintegrate in one or two hours unless properly fixed, and body cells show degeneration after the tissues have been left without fixation for a few hours, which changes may be interpreted as pathological.

Prepare a pint or quart of 5 to 10% formalin solution (2 to 4% formaldehyde) shortly before operation or autopsy. *Drop into the solution* slices of tissue, not more than ¼ inch thick, *as soon as cut.* Leave in the fixative for twenty-four hours or longer when the specimen is to be sent away to a laboratory for diagnosis. *The pathologist will attend to the other steps.*

We use two fixation solutions in routine work, one of 10% formalin and one of Zenker's solution. This latter requires prolonged washing of tissues following fixation and has little advantage over formalin for ordinary purposes.

Excision of Portion of New Growth for Diagnosis.—At one time the method of removing a small piece of a tumor for microscopical examination was recognized as

a proper procedure prior to complete removal of the growth. More recently the view has gained acceptance that such trauma of the tumor may aid metastasis to neighboring lymphatics and should not be practised. F. C. Wood takes exception to the view as to the danger involved in such diagnostic incisions and by experiments on a rat carcinoma which frequently metastasized to lungs, blood vessels and lymph-nodes showed that metastasis was not increased where a fragment was excised and the growth allowed to remain in situ for 10 or 12 days thereafter. The percentage of metastasis with diagnostic excision followed by removal was 22.2% while in the group of animals in which the tumor was removed without previous incision the percentage was 21.8. Where no operative procedures were instituted the percentage of metastasis was 32.2. These findings were obtained from experiments on 673 rats divided into the 3 groups noted above. The autopsies for recognition of metastasis were made about one month following operation and of the group 3 controls at the same time. He noted the danger of massaging tumors prior to or at operation as tending to promote metastasis.

Bloodgood, on the other hand, asserts that cutting into malignant disease certainly has an element of danger. According to him biopsy, if undertaken, should be conducted with thermal or chemical cauterization and in general a technic for the prevention of contamination of the wound with malignant tumor cells; that the type of operative procedure indicated should be ascertained by immediate frozen section, and undertaken forthwith; and that, if the operator is not prepared to obtain immediate microscopic diagnosis, and to proceed in accordance with the indications so ascertained, it is better for the patient that he perform a radical or complete operation for all lesions suggestive of malignancy.

Preparation of Tissue

Ordinary Method.—1. *Fixation.*—It is most important that the tissues to be examined be placed in the fixing fluid as soon after death or operation as possible. Degenerative changes are in this way avoided.

The piece of tissue to be fixed must not be too large. Using a sharp scalpel, or preferably a razor, a section of tissue about one-half inch square and not more than one-fifth of an inch thick should be dropped into the bottle containing the fixative. The bottom of this bottle should have a thin layer of cotton with a piece of filter paper covering it. There should be at least 20 times as great a volume of fixing fluid as of tissue to be fixed. Delicate tissues, as pieces of gut, should be attached to pieces of glass, wood, cardboard, or blotting paper before being placed in the fixative. In fixing certain specimens of tissue, especially pieces of slit intestine, it is a good plan to lay the specimen, while wet, peritoneal side downwards, on a piece of thick dry filter paper or lintless blotting paper; this prevents the specimen from being curled up. The whole is put into the fixing fluid, and the paper removed after fixation. The number or name of the specimen may also be written on the paper.

(*a*) Formalin. The most convenient fixative for the average medical man is: (1) A 10% solution of ordinary commercial formalin (4% of formic aldehyde gas), either in water or, preferably, in normal salt solution. Fixation is complete in from twelve to twenty-four hours. By placing in the incubator, at 37°C., two to twelve

hours in the formalin solution suffices. If fixed in the paraffin oven (56°C.), fixation is accomplished in about one-half hour. Formalin once used for fixation must be thrown away.

(*b*) Zenker's fluid. The fixative which probably gives the best histological pictures and with which we obtain the most satisfactory haematoxylin staining is Zenker's fluid. This is Müller's fluid, from which the sodium sulphate has been omitted, containing 5% of corrosive sublimate. It also contains 5% of glacial acetic acid, which latter is only added just before we are ready to fix the piece of tissue. Müller's fluid is:

Pot. bichromate	2.5 grams.
Sod. sulphate	1.0 gram.
Water	100.0 cc.

Zenker's fluid fixes in about twenty-four hours. After all corrosive sublimate fixatives we should wash the tissues in running water for twelve to twenty-four hours. The precipitate of mercury in the tissues is best removed by treating the section on the slide with Lugol's solution, rather than the tissue in bulk with iodine alcohol. A saturated corrosive sublimate solution in salt solution with the additional 5% of glacial acetic acid may be used as a substitute for Zenker's fluid.

(*c*) Alcohol. Where the tissue is to be examined chiefly for bacteria absolute alcohol is the best fixative. The piece of tissue should be small, not over $\frac{1}{5}$ inch thick, and is to be suspended by a string to the cork so as not to lie on the bottom where the alcoholic strength tends to become weaker. Better histological details are secured by fixing for two hours with 80% alcohol and then transferring to absolute for twelve to twenty-four hours.

(*d*) For fixation of neurological tissue see page 676.

NOTE.—Most laboratories prefer to receive tissue that has been fixed in formalin. Alcohol interferes with frozen sectioning and *postal regulations forbid alcohol in mail.*

2. *Dehydration.*—After washing for twelve to twenty-four hours in running water, following corrosive sublimate fixation, or simply washing for a few minutes after formalin, the tissues should be placed in 70% alcohol.

Tissues may be left in the 70% alcohol twelve to twenty-four hours and should then be transferred to 95% alcohol for an equal time. They are then transferred to absolute alcohol, where they remain from two to twelve hours and are then placed in xylol. The time in xylol should be as short as possible. So soon as the tissue looks clear it should be removed—thirty minutes to two hours.

Bolles Lee is a strong advocate of the superiority of cedar oil over xylol or any other clearing agent for paraffin imbedding. It does not affect delicate structures or make them brittle even when kept in the cedar oil for weeks or months. Furthermore, it does not matter whether the cedar oil is entirely removed before sectioning the paraffin as is the case for best results with xylol. Cedar oil will clear from 95% alcohol as well as from absolute alcohol.

3. *Imbedding.*—The tissue is now transferred to melted paraffin. Paraffin melting at 48°C. for winter work, and that melting at 54°C. for summer, are to be recommended. The time in the paraffin should not be prolonged. Two hours will ordinarily suffice. Some leave in the paraffin for twelve to twenty-four hours.

A medium with a melting point of 51°–53°C. which remains firm and tough at all temperatures is obtained by mixing one part of beeswax with five parts by weight of a mixture of hard and soft paraffin melting at 50°C., fusing them together, and filtering.

Next take a paper box (made of stiff writing-paper folded over a square of wood) and fill with the melted paraffin. As quickly as possible drop in the piece of tissue taken out of the paraffin bath with heated forceps and, so soon as the paraffin begins to solidify on the surface, place the paper box in ice water. When paraffin is rapidly cooled, crystallization is less.

The Acetone Method.—Take the tissues out of the 70% alcohol and place in acetone. After remaining in acetone for one or two hours, the tissues should be transferred to fresh acetone for an equal length of time. Dry calcium chloride in the bottom of the acetone bottles keeps it dehydrated. The pieces should then be placed in xylol for about one-half hour and then embedded in paraffin as directed above.

The Chloroform Method.—The procedure may be the same as in the method of passing through alcohols to xylol, substituting chloroform for xylol and then transferring to paraffin.

Where absolute alcohol is not obtainable, very satisfactory results may be obtained by transferring to a mixture of 95% alcohol and chloroform after immersion in 95% alcohol. Then going from the alcohol-chloroform mixture to pure chloroform, thence to paraffin.

Rapid Paraffin Imbedding Methods.—1. When a piece of tissue is not more than ¼ inch square and ⅛ inch thick, it is very easy to run it through in three to four hours. Thus:

Formalin 10% (57°C. incubator)	1 hour.
Acetone (room temperature)	1 hour.
Benzene (room temperature)	1 hour.
Paraffin (57°C. incubator)	1 hour.

Imbed and cool quickly in cold water.

2. *Method of Lubarsch.*—In this excellent method small pieces of tissue not more than ⅕ inch thick are placed in a wide test tube containing 10% formalin for ten to fifteen minutes, changing the fluid twice. Transfer to 95% alcohol ten minutes changing alcohol once. Absolute alcohol, for ten minutes changing twice. Pure aniline oil until tissues are transparent, fifteen to thirty minutes. Xylol, changing two to three times or until the xylol is no longer yellow, ten to twenty minutes. Imbed in paraffin for twenty minutes to one hour. During the entire process keep the test tube in a water bath or incubator at 50°C.

Imbedding in Celloidin:—

1. Alcohol 70%	12 to 24 hours
2. Alcohol 95%	24 hours
3. Alcohol absolute	24 hours
4. Ether and absolute alcohol equal parts	24 hours
5. Thin celloidin	24 hours to one or more weeks
6. Thick celloidin	24 hours to one or more weeks

7. Mount on blocks of vulcanized rubber.
8. Harden celloidin in chloroform for 1 or 2 hours followed by 80% alcohol.

Decalcification.—This is best accomplished by fixing in 10% formalin for twenty-four hours, then placing a small piece of the bone (not exceeding ½ inch square and ⅛ inch thick) in 5% sulphurous acid or 5% nitric acid.

This decalcifies in about two to seven days. Wash thoroughly in alkaline water and then in tap water. Pass through alcohols and xylol and imbed as before described.

Mitochondria.—Altmann in 1890 by means of new fixation methods, demonstrated intracellular bodies, granular or filamentar in shape, which have been variously termed "bioblasts," "symbiotes" and "mitochondria." They are known to occur in almost all active living cells. It has been repeatedly suggested that they are symbiotic organisms, but Cowdry and Olitsky, after careful study, conclude that they are not independent microorganisms.

In order to bring out these bodies, Altmann used fixation in equal parts of 5% potassium bichromate and 2% osmic acid, staining with aniline fuchsin and differentiating in picric acid. Ordinary fixatives also may be used, but mitochondria are preserved better in Regaud's fluid (4 parts of 3% pot. bichromate and 1 part formalin), Bensley's mixture (16 cc. of 2.5% pot. bichromate, 4 cc. of 2% osmic acid, 2 drops of acetic acid), or Bouin's fluid (15 cc. of sat. aq. picric acid, 5 cc. of formalin, 1 cc. of acetic acid). Tissues are mounted in paraffin. Sections may be stained by Giemsa's method, iron haematoxylin or fuchsin and methyl green.

Sectioning of Tissue

For sectioning it is necessary to have a good microtome. The best is that of Minot. Very satisfactory sections can be cut with the various types of student microtomes, costing from twelve to twenty dollars.

(In using a hand microtome, a razor with a flat edge is necessary. After experience, sections thin enough for histological but not for bacteriological examination can be made.)

If the piece of tissue is properly dehydrated and imbedded, thin sections (3 to 10μ) should be easily obtained, provided the knife be sharp. One advantage about the paraffin method is that it is only necessary to have a small part of the blade in proper condition. With celloidin the entire cutting edge must be perfect. Having cut the sections, they should be dropped on the surface of a bowl of warm water (45°C.). This causes the section to flatten out evenly.

Skin Sectioning.—Of all tissues that of skin offers the greatest difficulty in preparing sections. The best results can probably be obtained by fixation in picro-sublimate (saturated aqueous solution picric acid 1 part; saturated aqueous solution bichloride of mercury one part); to this stock mixture add 5% glacial acetic acid just before using. Fix small pieces of skin six to eighteen hours. Transfer direct to 70% alcohol in which the tissue may be kept indefinitely.

For sectioning run through alcohols to absolute and then to a mixture of absolute alcohol and carbon bisulphide (equal parts). Leave until tissue sinks, then transfer to pure carbon bisulphide until tissue sinks. Then transfer to a saturated solution of paraffin in carbon bisulphide and thence to paraffin. Bisulphide of carbon has the disadvantage of foul odor and inflammability but does not seem to render tissues brittle and difficult to section as does xylol.

Staining of Sections

Use of stains requires that section be affixed to slide and that the paraffin be removed. 1. Take a very small loopful of albumin fixative (white of fresh egg, 50 cc.; glycerin, 50 cc.; sodium salicylate, 1 gram) and deposit it on a cover glass. Now take up a loopful of 30% alcohol (1 drop of 95% alcohol and 2 drops of water) and applying it over the albumin fixative, smear out the mixture uniformly over the cover glass.

2. Pick up a section, from the bowl of warm water, on a strip of cigarette paper and apply it to the prepared surface of the cover glass. Blot with gentle pressure with a piece of filter paper over the strip of cigarette paper, and strip off this latter, leaving the section attached to the cover glass.

3. Now, turning the flame of the Bunsen burner down very low, or using a small alcohol flame, we hold the cover glass in a Stewart's forceps, section side up, over the flame and slowly lower it until the paraffin is observed to melt. This shows a temperature of about 50°C. The section is fixed by the coagulation of the albumin at about 70°C. To obtain this temperature lower the cover glass still more, and the moment vapor is seen to rise from the section the attachment of the section to the cover glass is assured.

4. Flood section on cover glass or slide with xylol; this dissolves out the paraffin. It is better to pour off the first xylol and drop on fresh xylol (one minute).

5. Remove xylol with two applications of absolute alcohol (one minute).

6. Treat specimen with two or three applications of 95% alcohol (one to two minutes).

7. Next wash in water (one or two minutes).

Staining is carried out as follows:

1. Flood specimen with haemalum or Delafield's haematoxylin (three to seven minutes).

2. Wash in tap water for about two to five minutes until a purplish tinge is developed in the section. The alkali in ordinary tap water develops this color. If the water is not sufficiently alkaline add about 10 drops strong ammonia to a tumblerful of water.

3. Apply 1 to 1000 eosin (aqueous) for thirty seconds to one minute.

4. Wash in water; then in 95% alcohol; then in absolute alcohol.

5. Apply a few drops of xylol and as soon as the section is perfectly transparent mount in balsam, or immersion oil.

The staining by haematoxylin and eosin is the best for the study of the histology of a section. It requires only about ten minutes to run a preparation through for diagnosis by this method.

The reagents are best kept in dropping-bottles.

The staining of sections on slides is exactly as for those on cover glasses. Coplin's staining jars are very convenient for use in staining slides.

Where the cover glass method is used, staining by Gram's method, acid-fast staining, capsule staining, etc., may be carried out as for bacterial preparations.

For staining Gram-positive bacteria in sections, the Gram method as for bacterial preparations, using dilute carbol fuchsin or Bismarck brown or safranin as a counterstain, gives good results.

For Gram-negative bacteria stain with thionin as for blood preparations (ten to twenty minutes). Then differentiate in 1 to 500 acetic acid solution for ten to twenty seconds, wash with water, then with 95% alcohol, and quickly through absolute alcohol and xylol. I prefer the panoptic staining.

Naval Medical School Routine Staining of Paraffin Sections on Slides.—Tissues fixed in formalin.

1. Fix specimen in 10% formalin for 24 hours, then cut into desired blocks 1/5 inch thick and about 1/2 inch square.
2. 95% alcohol six to twelve hours. Two changes.
3. Absolute alcohol six to twelve hours. Two changes.
4. Chloroform twelve hours (over night).
5. Paraffin bath two to five hours, 57°C.
6. Imbed—cut 3 to 6 microns thick.
7. Place sections in xylol 5 minutes.
8. 95% alcohol 5 minutes.
9. Float section on slide with aid of glass-rod lifter.
10. Remove excess of alcohol by wiping close to section.
11. Flood section with absolute alcohol from dropping bottle.
12. Drop on one drop of very thin celloidin.
13. Wipe off excess of celloidin close to section.
14. Haematoxylin (Harris) 5–15 minutes.
15. Wash in water.
16. Acid alcohol (1% HCl in 70% alcohol). Wash until section becomes rose color.
17. Wash in water.
18. Ammonia water (1.5%). Wash until section turns blue.
19. Wash in water.
20. Eosin (2% in 70% alcohol) one minute.
21. Alcohol 70%.
22. Alcohol 95%.
23. Absolute alcohol (two changes) dip.
24. Xylol. Two changes.
25. Mount in balsam.

Mallory's Eosin and Methylene Blue Stain.—This stain, used on paraffin sections of tissues fixed in Zenker's fluid, can be recommended as the very best general stain yet devised. It is a sharp nuclear stain, and, at the same time, brings out with a great deal of differentiation all the various other structures in the different tissues.

1. Stain paraffin sections in a 10% aqueous solution of eosin for one to two hours—in a paraffin oven.
2. Wash in water to get rid of the excess of eosin.
3. Stain in methylene blue for ten minutes—(methylene blue, 2 Gm.; alcohol 95%, 10 cc.; water, 90 cc.; just before using add 10 cc. of this methylene blue solution to 90 cc. of a 0.1% potassium carbonate solution).
4. Wash in water.
5. Decolorize and dehydrate in a 5% solution of colophonium (resin) in 95% alcohol. Keep section in constant motion so that the decolorization shall be uniform. Control the result under the microscope; when the pink color has returned to

the section and the nuclei are still deep blue, finish the dehydration quickly with absolute alcohol.

6. Xylol (two changes).

7. Mount in balsam.

For celloidin sections use 95% alcohol, blot, and pour on xylol; repeat the last two steps until the specimen is clear.

It is important to get a deep stain with eosin, because the methylene blue washes it out to a considerable extent. The eosin must be used first, because methylene blue is readily soluble in an aqueous solution of eosin, and therefore it is quickly extracted if the eosin is used after it, while on the other hand eosin is very slightly soluble in an aqueous solution of methylene blue which is precipitated by any excess of eosin.

Wolbach has found that the success of this staining method depends on the presence of colophonium in the alcohol used for differentiation. This is present in alcohol obtained from the barrel, but not in alcohol preserved in glass; it must, therefore, be added. This is most easily done by keeping on hand a 10% solution of colophonium in absolute alcohol, and adding a few drops of it to the alcohol in which the sections are differentiated. Wolbach has also shown that sections fixed in formaldehyde may be stained by this method, providing the amount of colophonium in the alcohol be increased from 5 to 10 per cent.

Van Giesen's Method.—Take of 1% aqueous solution acid fuchsin from 5 to 15 cc. Saturated aqueous solution picric acid 100 cc. The method of using is first to stain with haematoxylin in the usual way. Then pour on the picric-acid fuchsin solution and allow to stain for one to five minutes. Wash, pass through alcohols and xylol and mount in balsam.

Connective-tissue fibers, axis cylinders, and ganglion cells are stained a bright garnet red. Myelin, muscle fibers, and cells generally are stained yellow. Nuclear staining is that of haematoxylin. The stronger stain is used for nerve tissue; the weaker, for demonstrating connective tissue in tumors.

Demonstrating Treponema in Tissue.—1. *Levaditi's method.*—Take small pieces of tissue, about 2 mm. in thickness, and harden in 10% formalin for twenty-four hours and then in alcohol for the same period; then wash in water for a short period. They are stained in a freshly made solution of silver nitrate 1.5%, for three successive days, changing the solution each day, maintaining the blood temperature, and excluding light. The tissue is then placed in a 2% solution of pyrogallic acid, with the addition of 5% formalin. After remaining in this for twenty-four hours, light being excluded, they are passed successively through 85%, 95%, and absolute alcohol; imbedded in paraffin, and cut in about 5-micron sections. Equally good results may be obtained by allowing the silver nitrate to act at room temperature and imbedding in celloidin.

2. *Noguchi* has used the following modification in demonstrating spirochaetes in brain and cord: Fix ⅛-inch sections of tissue in 10% formalin for four or five days. Then place tissues in the following solution: Formalin 10 cc., pyridin 10 cc., acetone 25 cc., absolute alcohol 25 cc., distilled water 30 cc. Keep in this solution for five days at room temperature. Then wash in water for one day. Transfer to 95% alcohol for three days and then wash in water for one day. Put tissue in dark bottle in 1.5% aqueous solution of silver nitrate for five days at room temperature.

Wash in distilled water for five to six hours. Transfer to a reducing mixture of 95 cc. of 4% aqueous solution of pyrogallol and 5 cc. of formalin. Keep in this solution twenty-four hours. Wash in water and put through alcohol and xylol. Imbed in paraffin.

3. *Warthin and Starry's silver-agar cover glass method.*—See page 68.

Demonstrating Acid-fast Organisms in Tissue.—The following method of demonstarting acid-fast organisms in tissue has been used at the Hygienic Laboratory with exellent results.

1. Carbol fuchsin (Kinyoun's): ½ hour at room temperature.
   Basic fuchsin..... 4 Gm.
   Phenol crystals..... 8 Gm.
   Alcohol 95%..... 20 cc.
   Water to make..... 100 cc.
2. Wash in water.
3. Acid alcohol (1% HCl in 95 % alcohol) decolorize.
4. Wash in water.
5. Haematoxylin.
6. Wash in water.
7. Acid alcohol (1% HCl in 70% alcohol).
8. Wash in water.
9. Ammonia water (1.5%).
10. Wash in water.
11. Orange G (saturated aqueous solution) 10 minutes.
12. Pass quickly through 95% alcohol and two changes of absolute alcohol.
13. Clear in xylol.
14. Mount in balsam.

Tissue Stains.—*Harris' haematoxylin.*—This haematoxylin ripens immediately and is prepared according to the following formula:

Haematoxylin	1 gram
Alcohol	10 cc.
(dissolve the haematoxylin in the alcohol)	
Alum (ammonium or potassium)	20 grams
Mercuric oxide	½ gram
Distilled water	200 cc.

Dissolve the alum in the water by the aid of heat, and add the haematoxylin solution. Bring the mixture to a boil as rapidly as possible, and then add a half gram of mercuric oxide. The solution at once assumes a dark purple color. As soon as this occurs, remove the vessel containing the solution from the flame and cool by plunging at once into a basin of cold water. As soon as cool, the solution is ready for staining. This solution keeps for years in a well stoppered bottle (Harris).

The addition of 4% of glacial acetic acid increases the precision of the nuclear staining.

Delafield haematoxylin.—To make Delafield's haematoxylin, dissolve 1 gram of haematoxylin crystals in 6 cc. of 95% alcohol. Add this to 100 cc. of saturated

aqueous solution of ammonia alum. After exposure to light for a week, the color changes to a deep blue purple. Add to this ripened stain 25 cc. of glycerin and 25 cc. of methyl alcohol and, after it has stood for about two days, filter. The stain should be filtered from time to time as a sediment forms. This makes a stock solution which should be diluted 10 to 15 times with water when staining.

Romanowsky stain.—Staining sections with Romanowsky stains is not very satisfactory. The differential staining seems to fade out in passing through the alcohols. This may be avoided by blotting the section after staining and differentiation and then applying the xylol to the blotted section. After staining with Giemsa's stain for ten to fifteen minutes, differentiate with 1 to 500 acetic acid. When the section has a pinkish tinge, wash in water, dry, clear in xylol, and mount.

Good tissue staining may be secured with Wright's stain. After removing the paraffin with xylol and the xylol with absolute alcohol, pour on a sufficient number of drops of stain and after one minute dilute with an equal number of drops of water. Allow the diluted stain to remain for three to five minutes. Next wash in water; differentiate, until the tissue has a pinkish tinge, in 1 to 500 acetic acid. This differentiation is best done in a tumbler of the dilute acetic acid.

After washing in water, quickly pass through 95% and absolute alcohol, clear in xylol, and mount.

I now use the *panoptic method* for staining tissues. In this I stain with Wright's stain as given above but following the washing of the section we treat this with a dilute Giemsa (1–15) for ten to fifteen minutes. Then wash and differentiate in 1 to 1000 acetic acid in water in a small beaker. When the section assumes a pinkish tinge wash in tap water, then in 95% and absolute alcohol and clear in xylol. Then mount in liquid petrolatum, immersion oil or balsam.

Perl's test for iron in tissue.—Immerse a thin slice of tissue in a 5% solution of potassium ferrocyanide for five minutes then transfer to a 1% solution of hydrochloric acid. The presence of iron is shown by a Prussian blue color, which develops in about two minutes. This test applied to a section of skin is of diagnostic value in haemochromatosis. In pernicious anaemia the best results are obtained by using liver tissue.

Terry's rapid method of sectioning and staining fresh or formalin fixed tissue, using his neuiralized polychrome methylene blue stain. Frequently by this method tissue may be ready for microscopic examination in 30 to 60 seconds.

Method.—The piece of fresh or formalin fixed tissue is immobilized by pinning it to a cork board. With a very sharp razor blade wet with water a thin plane-parallel slice of tissue is cut, washed in water, drained and placed on one end of a glass slide. On the other end a drop of the neutralized polychrome methylene blue is spread out with a fine brush. Grasping an edge of the tissue with forceps, draw the section through the stain, care being taken to stain only the under surface. Fixed tissue usually stains in 1 to 2 seconds, fresh tissue in 2 to 4 seconds. The section is then quickly washed in water, placed on a slide stained side uppermost, covered with a cover glass and examined by transmitted light. For this purpose a 60 watt frosted Mazda lamp close to the mirror of the microscope is excellent.

Terry's five steps in making the stain are as follows: (1) Prepare 3 stock solutions A, B, and C. (2) Titrate stock solution A against C. (3) Alkalinize 100

cc. of B. (4) Polychrome 100 cc. of alkalinized B in 25 cc. quantities at 90 to 96°C. for 15 to 30 minutes. (5) With 1 cc. of C neutralize the A already added.

Steps in detail. 1. The three stock solutions in distilled water are:

A. 12% K_2CO_3, C.P., anhydrous	100 cc.
B. 1% Methylene blue, medicinal	1000 cc.
C. 10% Acetic acid, by volume	100 cc.

2. Titration.—Determine how much of solution A exactly neutralizes 1 cc. of boiling standard solution C using phenolphthalein as an indicator. Mark this quantity on bottle A.

3. Alkalinization.—Into a 100 cc. graduate place quantity of A equivalent to 1 cc. of C. Add enough of B to make 100 cc. and mix thoroughly.

4. Polychroming.—25 cc. quantities are placed in each of four one ounce bottles. These unstoppered bottles are placed in water which is brought to a boil in about 10 minutes. Note the time and remove the bottles one by one, 15, 20, 25, and 30 minutes later. Let them cool slowly. The water should be kept boiling while the bottles are in it.

5. Neutralization.—To each 25 cc. of polychrome stain add 0.25 cc. of solution C.

The staining solutions keep well. Filtration is usually unnecessary and should not be carried out immediately. The four bottles are polychromed differently to permit each worker to make and choose stains which best suit his own taste, light and work.

Neuropathologic Technic

Many special methods have been devised for the histopathologic examination of the nervous system. For routine purposes formalin fixation, paraffin embedding and haematoxylin-eosin staining give good results. Special methods are required however for more detailed study of (1) nerve cells, (2) nerve fibers and neurofibrils, (3) myelin sheaths, (4) neuroglia cells and fibers, (5) connective tissue, (6) fat.

The material from necropsy is fixed in 10% formalin solution preferably made isotonic with sodium chloride (0.9%) and kept neutral with an excess of magnesium carbonate. The maintenance of neutrality is imperative if cell stains are desired upon old material. At the time of necropsy small blocks may be cut and fixed in formol-bromide solution for neuroglia impregnation, or in Carnoy's solution for the demonstration of nerve fibers. If a tumor is present, fix a small piece in Zenker's fluid. It is better when possible to put off sectioning of the brain until after fixation in toto. Relationships may thus be maintained even after the brain is sectioned.

The most important sections to take after fixation of the whole brain are the frontal, precentral and hippocampal cortex, a strip from the insula to the third ventricle taking in all the basal ganglia (such a strip from a brain cut in frontal sections may be mounted on an ordinary slide), the midbrain, dentate nucleus of the cerebellum with some of the neighboring cortex, the medulla oblongata just below the pons, and the upper end of the spinal cord. Sections may be labelled by writing directly upon the slightly dried surface with pen and ink.

Without previous washing the small pieces are placed directly in 80% alcohol 24 hours or longer, 95% alcohol over night, absolute alcohol 4 to 6 hours, cedar oil

over night or longer until the sections are clear, chloroform 3 hours, paraffin in the 55 degree oven 2 to 4 hours depending upon the thickness of the block and the amount of paraffin used. Long periods in strong alcohol, chloroform and the oven shrink brain tissue badly. The blocks are then embedded, cut and the sections fixed to slides with albumen glycerin solution. Three slides may be made from each block, one for haematoxylin-eosin, one for cell stain, and one for glia stain. If the tissue is obtained more than 12 hours after death, the glia stain is practically worthless.

Special Staining Methods

(1) **Cell Stain (Sheldon-Dyar).**—Deparaffin slides and carry through graded alcohols to water. Stain in the following solution over night at room temperature:

Azure C (Monomethyl thionin)*	0.20 Gm.
Erythrosine	0.10 Gm.
Water	100. cc.

* Azure C is at present being made by the Color Laboratory, U. S. Bureau of Standards, Washington, D.C. and will probably soon be marketed by the National Aniline Chemical Company.

The azure is dissolved in 50 cc. water. The erythrosine is dissolved in 5 cc. 95% alcohol and added to 45 cc. water. The two solutions are mixed and filtered. More erythrosine must be added after using the stain a few times. Filter the stain each time before using.

The excess of blue is removed with absolute methyl alcohol 10 seconds and the section then differentiated in

Absolute ethyl alcohol	50 cc.
Glacial acetic acid	10 drops

watching the process under the microscope until the background is pale pink and the nerve cells begin to fade. Wash thoroughly with absolute alcohol, clear in oil of bergamot and then in xylol; mount in neutral balsam or thick cedar oil. The body and dendrites of the nerve cells are violet with blue tigroid bodies, the nucleus colorless, the nucleolus almost black. Glia nuclei are pale blue, endothelial nuclei navy blue. Degenerating nerve cells lose their tigroid bodies and their concave outlines, or become shrunken, twisted and hyperchromatic in the chronic stages. Old nerve cells show an accumulation of yellowish lipoid pigment.

Nissl's method using either thionin or Giemsa staining gives good results in demonstrating axonal reactions or other degenerative changes in nerve cells, as shown by bulging of the concave sides of the cells, eccentric nucleus and granular appearance of the tigroid bodies.

(2) **Nerve Fibers and Neurofibrils (Bielschowsky).**—Thinnest possible frozen sections from formalin fixed material are carefully washed in distilled water to remove all the formalin. If a crystal of thymol is added to the distilled water to prevent bacterial growth the sections may be kept there almost indefinitely. With a glass needle carry sections into 2.5% silver nitrate 40–48 hours. Keep in dark. Rinse sections rapidly in distilled water and carry into the following solution:

Silver nitrate crystals..... 1 Gm.
Distilled water..... 20 cc.
Add
Sodium hydroxide (40%)..... 5 drops

Almost dissolve the precipitate by adding strong ammonia water drop by drop stirring vigorously. Avoid any excess of ammonia. Dilute to 25 cc. and filter. Sections should turn dark in from 3 to 10 minutes.

Wash hurriedly in distilled water and reduce in 20% formalin.

Wash in distilled water and tone in the following solution.

Gold chloride (1% solution)..... 3–10 drops
Glacial acetic acid..... 2– 3 drops
Distilled water..... 20 cc.

until the brown color has given place to gray or violet.

Wash in water and fix in 5% sodium hyposulfite solution. Wash thoroughly. Dehydrate the section on the slide, clear with xylol and mount in balsam. The intracellular neurofibrillae and nerve fibers are black against a grayish or violet background.

To dehydrate a frozen section on the slide, coat the slide with albumen-glycerine and dry quickly over a flame. Mount the section from water on the slide and blot with filter paper. Pour on 95% alcohol and blot again. Flood twice with absolute alcohol, blotting each time, then with xylol twice or until the section is transparent. Blot and mount with balsam before the section dries.

If the tissue is very soft it may be soaked in 10 to 20% gelatin solution in the 37° incubator for an hour or more and then allowed to cool. The block of gelatine with the tissue inside may then be cut directly by freezing, but it is better to fix the gelatine in 10% formalin over night, after which the block is trimmed and then cut on the freezing microtome.

For the demonstration of nerve fibers in various organs of the body, in tumors, in the skin, etc., impregnation is carried out upon the block of fresh tissue.

Silver Impregnation of Nerve Fibers in Blocks (Campbell).—Fix blocks not over 3 mm. thick for three hours in Carnoy's fluid:

Absolute alcohol..... 30 cc.
Chloroform..... 15 cc.
Glacial acetic acid..... 5 cc.

Wash in several changes of absolute alcohol for 24 hours, and transfer to 50% alcohol or six hours. Place for 24 hours in:

50% alcohol..... 50 cc.
Ammonia water..... 5 drops.

Rinse quickly in distilled water and impregnate in a 2% silver nitrate solution for 5 days. Then wash in several changes of distilled water for 1–2 hours, and reduce for 24 hours in:

Hydroquinone..... 1 Gm.
Distilled water..... 100 cc.
Neutral 40% formaldehyde..... 15 cc.

Wash in several changes of distilled water 2–3 hours, dehydrate and embed. The impregnation and reduction should take place in the dark at 37°, the final washing in the dark at room temperature. After the sections have been cut they may be counterstained. Medullated nerve fibers appear brownish, non-medullated nerve fibers and end-plates black.

Silver Impregnation of Nerve Fibers in Paraffin Sections (Freeman).—Paraffin sections of nerve tissue preferably fixed in alcohol (though the method is applicable after formol fixation) are run through xylol and graded alcohols to water. The slides are then laid, section side up, in dishes containing warm 10% gelatine solution which should cover the section by 3 to 5 mm. After solidification of the gelatine, an amount of 2% silver nitrate solution equal to the volume of gelatine solution used is allowed to run onto the surface. The gelatine layer acts as a tissue of uniform thickness and consistency through which the silver ions can diffuse. The plates are kept in the dark at room temperature for 2 to 6 days. The supernatant fluid is then discarded and the gelatine melted by immersion of the plate in hot water until the gelatine disc slides away. Without previous washing then, the sections are reduced in the "developer" used by Warthin & Starry to demonstrate spirochetes:

Silver nitrate (2%)	3 cc.
Warm glycerin	5 cc.
Warm 10% gelatin	5 cc.
Warm 1.5% agar agar	5 cc.
Hydroquinone (5%)	0.5–1.0 cc.

Shield from direct sunlight. Too much hydroquinone results in quick development and loss of contrast; too little in precipitation of silver. Nerve fibers are black on a brown background.

(3) **Myelin Sheaths.**—Myelin is a lipoid found in the sheaths of nerve fibers in both the central and peripheral nervous systems. Ferric and chromic salts mordant the myelin so that it takes the hematoxylin stain. When the nerve fiber is interrupted, both fiber and sheath degenerate in the distal direction. In doing so the myelin is converted partly into fat which is gradually carried away by scavenger cells. It begins to appear about five days after the injury and can still be found after a lapse of several months. Only in the late stages of nerve degeneration is the Weigert stain (and its many modifications) of value and then it gives a negative picture, i.e. does not stain the degenerated fibers because the myelin has disappeared. The method is very useful however, because nerve fibers in the central nervous system never regenerate. For the detection of recent degenerations see (6) fat stain.

Weigert-Pal Method.—Thin slices of tissue are fixed in 10% formalin for about four days. The tissue should then be transferred to 5% potassium bichromate for about twelve days. The tissue is then imbedded and sections cut. If only recently mordanted these sections may be at once stained with Weigert's haematoxylin for twelve to twenty-four hours (10 cc. ripened 10% solution haematoxylin in absolute alcohol and 90 cc. water). Wash in water to which about 2% of a saturated solution of lithium carbonate has been added. Now differentiate from one-half to five minutes in 0.25% solution of potassium permanganate until the gray matter looks a brownish-yellow. Next treat sections with oxalic acid 1 gram, potassium sulphite 1

gram and water 200 cc. until the gray matter is almost colorless. This takes only a few seconds. Wash in water, pass through alcohols and xylol and mount in balsam.

Frozen Section Method for Myelin Sheaths (Spielmeyer).—For small blocks this method is replacing the more cumbersome celloidin methods. Weigert sections are unsatisfactory after paraffin embedding.

Formalin fixed material is cut by freezing, at 30 microns. After washing in water the sections are mordanted for six hours in:

Ferric ammonium sulphate (iron alum)	2.5 Gm.
Water	100 cc.

Wash in water, then treat with 70% alcohol for 10 minutes.

Stain over night at room temperature or 4 hours at 37° in:

Ripened 10% haematoxylin in absolute alcohol	1 cc.
Water	20 cc.

Wash in water and differentiate in 2.5% ferric alum until the gray matter is yellowish and the white matter remains dark blue. Wash thoroughly in water, dehydrate on the slide and mount in balsam.

(4) **Neuroglia Cells and Fibers.**—The neuroglia is ectodermal connective tissue and resembles ordinary connective tissue in its behavior. Three main types are distinguished: (a) Fiber-forming; (b) Oligodendroglia; (c) Microglia. There is fairly good evidence now that the microglia cell are identical with the wandering cells of connective origin.

(a) The fiber-forming glia cell has a round vesicular nucleus, a small amount of cytoplasm that is stained with difficulty, and numerous wavy processes, the heaviest of which ends on the wall of a vessel in a small expansion called the glia-foot. These cells form the fibrous scar-tissue after an injury, inflammatory process or vascular disturbance. The fibers stain with phosphotungstic acid-haematoxylin, the cell bodies and fibres by Cajal's gold-sublimate impregnation.

(b) Oligodendroglia cells have few processes and almost no cytoplasm. They are observed as small compact nuclei in the vicinity of nerve cells and in the white matter.

(c) The microglia cells are scavengers, with small elongated nuclei and polar processes. Actively ameboid, they take up products of degeneration (fat, myelin bodies, pigment) become greatly swollen (compound granule cells, gitter cells) and migrate toward the sheaths of the vessels. The fat laden ones are well seen in Scharlach R preparations. The ordinary cells may be impregnated selectively by the method of del Rio Hortega.

Neuroglia fibres disintegrate within a few hours after death so that no method is of much use when the interval between death and fixation of tissues is more than 12 hours. Fresh warm tissues respond best.

Neuroglia Fibers.—Phosphotungstic acid-haematoxylin staining is best after Zenker fixation, but can be done on tissues fixed in formalin or alcohol if the section on the slide is mordanted with Zenker's solution 1 hour, followed by Lugol's solution 10 minutes and then by alcohol. After washing in water the slide is flooded with potassium permanganate solution (0.25%) for 5 minutes and then placed in 5% oxalic acid solution 5 minutes. After careful washing the slides are stained over night in:

Haematoxylin	0.1 Gm.
Water	80. cc.
10% aqueous solution of phosphotungstic acid	20. cc.

Next day the sections are differentiated without previous washing, in 95% alcohol. Dehydrate and mount.

Neuroglia fibers and myelin sheaths are blue, connective tissues red.

Neuroglia Cells (Fiber-forming) (Cajal).—Special sections, taken at the time of necropsy, are fixed in:

Formalin, 40% C.P. neutralized with chalk	15 cc.
Ammonium bromide, C.P.	2 Gm.
Distilled water	100 cc.

After 2 to 10 days frozen sections are cut at 20 microns and received in fresh formol-bromide solution. After brief rinsing in distilled water they are placed in:

Gold chloride (dark brown acid variety) 1% solution	10.0 cc.
Mercuric chloride *crystals* (not powder)	0.5 Gm.
Distilled water	60.0 cc.

(Dissolve sublimate by heat, filter, cool and add gold solution.)

The dishes with the sections spread out flat and not overlapping are kept in the dark for 4 to 6 hours at 18 to 22°C. The temperature is very important. Wash sections in distilled water, fix well in 5% sodium hyposulfite solution, wash again, dehydrate on the slide, clear in xylol and mount in xylol-balsam. Clean glassware and glass needles are essential.

The neuroglia cells and their prolongations are black against a purplish background.

(5) **Connective Tissue Stain (Mallory).**—The best results are obtained by fixing blocks of tissue (either fresh or previously fixed in formalin) in Zenker's fluid, for 12 to 24 hours. Wash 24 hours in running water, dehydrate, embed, cut and mount the sections on slides. Mordanting may be carried out on the sections but the results are inferior. Deparaffin, carry through absolute alcohol to 95% alcohol, then into Lugol's solution to remove the mercury precipitated in the tissue, 10 minutes. Remove the iodine with alcohol, wash in water and stain 5 to 10 minutes in 0.2% aqueous solution of acid fuchsin. Then transfer directly to the following solution for 20 minutes or longer:

Aniline blue, water soluble	0.5 Gm.
Orange G	2.0 Gm.
1% phosphomolybdic acid in water	100.0 cc.

Wash in water, dehydrate, clear in xylol and mount. The connective tissue is bright blue, nuclei red, erythrocytes orange.

(6) **Fat Stain.**—Recent degenerations are recognized by fat stains. Fat and myelin both reduce osmic acid, but when the myelin is mordanted with chrome salts (the Marchi method) only the fat is stained black. The Marchi method is delicate but difficult and limited in its application by shallow penetration of the osmic acid. Candle scarlet and Scharlach R are taken up by unsaturated fats (oleic and palmitic, not stearic), staining them bright scarlet. Myelin takes a magenta stain.

In place of the uncertain and lengthy Marchi method, the ordinary fat stain on frozen sections is recommended.

Fat Stain (Herxheimer).—Frozen sections at 10 to 20 microns are placed for 15 seconds to 1 minute in:

70% alcohol	50. cc.
Acetone	50. cc.
Candle scarlet	0.25 Gm.
or Scharlach R	0.5 Gm.

The stock bottle must be tightly stoppered and the staining dish covered to prevent evaporation and precipitation of the stain in crystals on the section. Filter the stain before using it.

Remove the excess of stain by short washing in 70% alcohol, then wash in water. Counterstain in weak alum-haematoxylin solution (1–3), differentiate in water with a few drops of ammonia, then wash in tap water for several minutes, mount on the slide, blot with filter paper and mount in glycerin or Farrant's medium. The blue counterstain furnishes a delicate contrast to the red fat globules.

Farrant's medium:

Gum arabic	120 Gm.
Distilled water	120 cc.

After 12 hours add 60 cc. glycerin and mix thoroughly. Filter through cotton and add a crystal of thymol for preservation.

Marchi's Method.—Small pieces of nerve tissue are hardened in Müller's fluid for seven to ten days, and are then transferred to a mixture of two parts Müller's fluid and one part of a 1% osmic acid solution and should remain in this mixture for about seven days. The tissue thus treated is run through alcohols and imbedded in paraffin in the usual way. With Marchi's method only the oleic acid of fatty degeneration is stained.

The method is not useful until three or four days have elapsed from the onset of the conditions causing the degeneration and it is applicable for only three or four months because by that time phagocytes have taken up the pathological fat which is stained in the Marchi method.

The Technique of Making and Staining Frozen Sections

An automatic laboratory microtome of the Spencer type with a carbon dioxide freezing attachment furnishes a practical and a very satisfactory means of making frozen sections. This type of microtome can also be used for paraffin or celloidin sectioning. The microtome is supported by means of a bracket above the work table and is connected by means of the freezing attachment to the carbon dioxide tank beneath the table. Frozen sections may be made from either fresh or fixed tissue.

Fresh Frozen Tissue Sectioning.—This is invaluable as an aid in diagnosis at time of operation. Polychrome methylene blue is usually recommended as the proper stain for fresh frozen sections, but saturated aqueous solution of thionin is also excellent and is more easily prepared.

Technique of Wilson.—Freeze bits of tissue 2 × 10 × 10 mm. in dextrin solution (dry dextrin stirred in boiling water to consistency of maple syrup) and cut 5 to 15 microns thick. Remove sections from knife with tip of finger and allow them to thaw thereon. Unroll sections with camel's hair brush in 1% sodium chloride solution. Stain 10 to 20 seconds in polychrome methylene blue. (See Terry's method of polychroming, p. 675.) Wash momentarily in fresh 1% sodium chloride solution. Mount in Brun's glucose medium (glucose 40 Gm., camphorated spirit 10 cc., glycerin 10 cc., distilled water 140 cc.).

Fixed Frozen Tissue Sectioning.—Tissue should be fixed for at least 12 hours. Any fixative may be used, preferably 10% formalin. Tissues fixed by alcohol or any other reagent, except formaldehyde, must be washed in running water for some hours before they can be frozen. Even in case of a tissue fixed in formaldehyde, washing in water for ten to thirty minutes is advisable, as better sections can be obtained.

The Naval Medical School routine for sectioning and staining fixed frozen tissue.—

1. Cut specimen into desired blocks 5 × 5 × 3 mm.
2. Fasten to freezing plate by means of dextrin solution.
3. Freeze and cut sections not more than 10 microns thick.
4. Remove section from knife blade with finger and place in distilled water.
5. Float section on slide with aid of glass-rod lifter.
6. Remove excess of water by wiping close to section.
7. Flood section with absolute alcohol from dropping bottle.
8. Drop on one drop of very thin celloidin.
9. Wipe off excess of celloidin close to section.
10. Haematoxylin (Harris; see p. 674), 5–15 minutes.
11. Wash in water.
12. Acid alcohol (1% HCl in 70% alcohol). Wash until section becomes rose color.
13. Wash in water.
14. Ammonia water (1.5%). Wash until section turns blue.
15. Wash in water.
16. Eosin (2% in 70% alcohol).
17. Alcohol 70%.
18. Alcohol 95%.
19. Absolute alcohol (two changes) dip.
20. Xylol (two changes).
21. Mount in balsam.

Sections cut and stained by this method are permanent and will last for years. Satisfactory results can be obtained with decalcified bone tissue. For Gram's, acid-fast, and silver nitrate staining better results are obtained by using albumin in preference to celloidin in fixing section to slide.

B—MOUNTING AND PRESERVATION OF PATHOLOGICAL SPECIMENS AND ANIMAL PARASITES

To Mount Small Round Worms.—Wash the hookworm, whip-worm, or filaria in salt solution, then drop in 70% alcohol containing 5% of glycerin, the glycerin-alcohol mixture being at a temperature of 60°C. When cool, pour into Petri dishes and allow the alcohol to evaporate in the 37°C. incubator.

Mount in glycerin jelly, preferably in a concave slide, and ring the preparation with gold size. The following is the formula for Kaiser's glycerin jelly: Soak 1 part of gelatin in 6 parts of distilled water for two hours. Then add seven parts of glycerin. To the mixture add 1% of carbolic acid, warm for fifteen minutes, with constant stirring, and then filter through cotton.

To Prepare Tape-worms.—Wash in salt solution. Wrap around a piece of glass as a glass slide and fix in salt solution containing 2 to 5% of formalin. Then keep the preparation permanently in 70% alcohol. If preferred, the specimen may be run through alcohols and xylol and mounted in balsam.

Larvae.—Mosquito larvae may either be prepared as are small round worms or they may be dropped into 70% alcohol at 60°C. and then passed through alcohols and cleared in xylol and mounted in balsam. Flukes and insects may require treatment with hot (60° to 70°C.) solution of 10 to 20% sodium hydrate solution. Then wash thoroughly in water and subsequently pass through alcohols to xylol and mount in balsam. Clove oil or cedar oil clears more slowly, but makes specimens less brittle than does xylol. Another satisfactory method is to drop insects or larvae into acetone at 60°C. and after being in this from one to twelve hours to clear in xylol or clove oil and mount in balsam.

Nematodes.—Looss has a method of first washing a small nematode or delicate fluke in salt solution. Then pouring this first salt solution out of the test tube in which the washing was carried out, add fresh salt solution, and then an equal amount of saturated aqueous solution of bichloride of mercury. The shaking is easily carried on in the test tube. After washing in water the worm is passed through alcohols, one strength of which should contain iodine. Clear in xylol and mount in balsam.

The following method gives very satisfactory results with hookworms:

1. Specimens preserved in 70% alcohol:

(*a*) Place directly in watch glass of carbol-xylol.

(*b*) Observe on stage of dissecting microscope until completely cleared (twelve to twenty-four hours).

(*c*) Mount in chloroform balsam (saturated solution).

2. Specimens preserved in 10% formalin:

(*a*) Dehydrate in absolute alcohol five to fifteen minutes.

NOTE.—Care should be taken not to completely dehydrate the specimens else they will appear shrivelled and distorted.

(*b*) Place directly in watch glass of carbol-xylol.

(*c*) Observe on stage of dissecting microscope until completely cleared (twelve to twenty-four hours).

(*d*) Mount in chloroform balsam (saturated solution).

An excellent method is that of Langeron: After washing in salt solution fix for a few hours in 5% formalin. Then transfer to lactophenol which has been diluted with an equal amount of water. Allow to remain in this solution for several hours and then transfer to pure lactophenol in which fluid the specimens are to be mounted. Ring with paraffin or with gold size. (To make lactophenol take 2 parts of glycerin and 1 part each of distilled water, crystallized carbolic acid and lactic acid.)

A quick method of preparing small nematodes for examination is to fix them for from two to twelve hours in 5 to 10% formalin, this being heated to 60°C. at the time the worms are dropped into it. Then transfer to the following solution:

Glucose syrup (glucose, 48; water, 52)	100 cc.
Methyl alcohol	20 cc.
Glycerin	10 cc.
Camphor, q. s. (a small lump for preservation).	

They may be mounted directly in this and the cover slip ringed with about 60°C. paraffin, followed with gold size.

Preparations so cleared and mounted in glycerin jelly should be also ringed with paraffin or some cement.

Flukes, cestodes, and nematodes are best stained with carmine. The following is a good formula: Dissolve, by boiling, 4 grams carmine in 30 drops HCl and 15 cc. water. Then add 95 cc. of 85% alcohol and filter while hot. Neutralize with ammonia until precipitate begins to form. Then filter cold.

1. Stain parasites taken from 70% alcohol for five to twenty minutes. 2. Differentiate in 3% hydrochloric acid. 3. Pass through alcohols to xylol and mount in balsam.

Mites, Fleas and Various Small Insects.—By simply taking 1 or 2 drops of liquid petrolatum and mounting the specimen in it, then covering with a cover glass, one is able to study the details of these objects almost as well as if they were passed through acetone and xylol into balsam. Liquid petrolatum is also most excellent for mounting the aërial hyphae of fungi with their sporangia as well as for Romanowsky-stained blood smears.

Preservation of Stools to be Examined for Ova.—During the war, thousands of specimens of stools from all parts of the world were sent to the Naval Medical School for examination for evidence of hookworm and other intestinal parasitic diseases. The following method proved very satisfactory, the ova being well preserved for months.

1. Collecting: The method of collection used by Siler and Cole, U. S. Army, is recommended. Sheets of paper 14 by 14 inches are placed over each of a dozen cheap wash basins on the latrine floors. Specimens are taken from faeces deposited on the paper, and placed in a bottle. The paper and the tongue depressor used in sampling can then be thrown into the latrine and a fresh piece of paper placed over basin.

2. Preservation: It is recommended that each specimen bottle be half filled with 10% solution of formalin (4% solution formaldehyde). From three portions of the stool, sufficient faeces should be taken with the tongue depressors to fill the bottle almost full. Emulsify the faeces in the formaldehyde solution at once. Replace the cork securely and mark plainly on the adhesive strip holding the cork in place, the name and rate of the patient.

Pathological Specimens.—Pathological tissues which are to be sent to a laboratory for sectioning or to be kept for future study should be fixed by one of the methods given in Section A of the appendix.

Formalin fixation is the more convenient—that with Zenker's fluid the more perfect. After fixation with Zenker's fluid the pieces of tissue must be washed in running water over night.

After fixation the pieces of tissue are transferred to 70% alcohol in which they may be kept indefinitely.

For preservation of gross specimens the method of KAISERLING is generally used. Fix for from one to five days in Solution 1:

Formaldehyde	200 cc.
Water	1000 cc.
Nitrate of potassium	15 grams.
Acetate of potassium	30 grams.

The position of the specimen should be changed from day to day. The volume of fluid used should be at least five times that of the specimen. Drain and transfer to 80% alcohol for a few hours, then into 95% alcohol until the natural color is just restored.

Finally preserve in solution II:

Acetate of potassium	200 grams.
Glycerin	400 cc.
Water	2000 cc.

It is advisable to keep these specimens in the dark as light destroys the natural color.

To Prepare Flies or Mosquitoes for Transmission through the Mails.—Wrap the insect carefully in a piece of tissue paper (toilet paper answers). Moisten sawdust with 5% carbolic acid solution and fill around the tissue paper in the box containing them.

It is very satisfactory to take a tube-form vial with a cork from the inner surface of which two small shallow holes have been bored, one containing paraformaldehyd, the other camphor. The insect is mounted upon a pin stuck in the cork, which latter is inserted and paraffined externally.

C—EQUIVALENT-NORMAL SOLUTIONS

An equivalent-normal solution contains the hydrogen equivalent of a substance, expressed in grams, dissolved in sufficient distilled water to make 1 liter. The hydrogen equivalent is the number of grams that will unite with 1 gram of hydrogen or its equivalent. For an acid, the hydrogen equivalent would be the molecular weight divided by the number of *replaceable* hydrogen atoms that it contains. For a base, it would be the molecular weight divided by the number of hydroxyl (OH) groups.

To make a normal (indicated by N) solution, dissolve in distilled water the proper amount of the substance, and make up the volume to exactly 1000 cc. Thus NaOH has one hydrogen equivalent: Na = 23, O = 16, and H = 1, so one dissolves 40 Gm. NaOH in distilled water, and makes the volume up to exactly 1 liter. Again, oxalic acid has the formula $(COOH)_2.2H_2O$, with a molecular weight of 126; containing two carboxyl groups, it has two hydrogen equivalents and it is necessary to divide by 2. So we dissolve 63 Gm. in water, and make up to 1 liter.

Preparation.—If a chemical laboratory is not accessible, one may prepare such solutions with an error so slight as to be unimportant in clinical work in the following manner: Select perfect crystals of oxalic acid, such as can be obtained in a drug store, and weigh out, on the most accurate apothecary scales available, 6.3 Gm. of the most perfect crystals in the bottle. Put these preferably in a volumetric flask, and make up with distilled water to 1 liter. Less accurate is the use of a measuring cylin-

der. With care, this method should give N/10 oxalic acid in which the error is less than 1%.

NaOH being very hygroscopic, it is impossible accurately to prepare a normal solution directly by weighing the solid. Having N/10 acid at hand, an N/10 NaOH solution may be prepared by weighing an excess of the substance, about 5 Gm. stick caustic soda, and dissolving it in about 1100 cc. distilled water. By means of a pipette, place 10 cc. in a beaker, and add 6 drops phenolphthalein solution (1% in 95% alcohol). Fill a burette with N/10 oxalic acid, and run it into the NaOH solution until the violet-pink color is just discharged. Reading the number of cubic centimeters of N/10 acid used, we can calculate the strength of the NaOH solution. It is well to repeat the titration, and use an average. If 10.5 cc. of oxalic acid solution were required, it would show that the NaOH solution was stronger than N/10, as only 10 cc. would have been necessary if it had been of N/10 strength. It is, therefore, necessary to dilute in the proportions of 10 : 10.5. To do this, take exactly 1000 cc. of the too concentrated NaOH solution, add 50 cc. distilled water to it, mix thoroughly, and there is then 1050 cc. of N/10 NaOH. Calculation: 10 : 10.5 = 1000 : x.

It is sometimes desirable to use *carbonate-free NaOH*, and this may easily be prepared by dissolving 100 Gm. C. P. NaOH in 100 cc. distilled water. The NaOH dissolves completely, but any Na_2CO_3 present is insoluble. Let it settle, or it may be centrifuged. Of the clear supernatant solution obtained after sedimentation, 55 cc. dissolved in CO_2-free water sufficient to make 1 liter will give approximately N/1 NaOH. Standardize in the usual way. Such a solution should, of course, be protected from the CO_2 of the air by storing in a paraffin-lined bottle, the 2-holed rubber stopper of which bears guard tubes, and a glass tube for siphonage, the latter being lined and coated with paraffin. The guard tubes comprise one containing sodalime (or NaOH solution) and one with H_2O, and are arranged in series so that, when siphonage is established, the sodalime removes CO_2 from the incoming air, and then the H_2O prevents the carrying of any aklali into the bottle.

Water can be freed from CO_2 by vigorous boiling for 15 to 20 minutes (not longer), or by aeration for several hours, the air sucked through having been passed through NaOH solution (or sodalime tubes) and water. If the water is exposed to the air, CO_2 will again be absorbed, so, if stored, it should be protected by the method described for the NaOH solution above, although the paraffin lining of tube and bottle is not essential.

Since Acidum Hydrochloricum, U.S.P., is about two-thirds water (68.1%), in order to make N/10 HCl, which would require 3.65 Gm. absolute acid per liter, it is necessary to take about three times this amount of U.S.P. acid. Take 12 cc., and add distilled water to make 1100 cc. Place 10 cc. in a beaker, add phenolphthalein solution, and titrate. If 11 cc. of N/10 NaOH were required, it would be necessary to add 100 cc. water to 1 liter of the diluted acid. Calculation: 10 : 11 = 1000 : x. Other acid and alkali solutions can be made in the same way as are those described.

D—HYDROGEN-ION CONCENTRATION

Hydrogen-ion concentration has assumed such an important position in biological studies that I will briefly review its salient facts. Medical men are especially concerned with its relations to bacteriology and the questions of enzyme, or ferment,

activity, acid-base equilibrium, and general metabolism in the human body. Most chemical compounds partially dissociate into ions when placed in solution, especially in water, and when we use the term hydrogen-ion concentration, we mean the weight (in grams) of the hydrogen *in ionic form* present per liter of solution. The extent of this dissociation depends upon the nature of the compound, the nature of the solvent, the temperature, the dilution, etc. The usual titration of volumetric analysis will determine the amount of a substance present, and then its total hydrogen can easily be calculated if the formula of the compound is known. The dissociated (ionic) hydrogen, however, comprises ordinarily only a portion of this total hydrogen, and the determination of its amount is difficult unless special methods be employed, viz., the electrometric or the colorimetric. The electrometric method is the more accurate, but it involves expensive and delicate apparatus. For our purposes, the colorimetric method, which employs buffer solutions and indicators, is quite satisfactory.

Living cells being influenced by the acidity due to ionic hydrogen (H^+) and by the alkalinity due to ionic hydroxyl (OH^-), rather than by total hydrogen or hydroxyl, it devolves upon us in our study or control of their vital phenomena to see that they are provided with a suitable environment, i.e., one in which the ions are in the desired concentration.

pH.—Concentration may be variously expressed. Brackets, as [HCl], [H^+] indicate that the concentration of the bracketed species is referred to. The same is indicated in the functions C_{H^+} and C_{Cl^-}. The concentration may have any value so long as a consistent system of units is maintained. The unit generally employed is the gram-molecular weight per liter—molar concentration.

Concentration may also be expressed in terms of normality, as 0.1N, N/10, etc., or in terms of mols (a mol, or gram-molecular weight, is the molecular weight expressed as grams), as 0.1M, M/10, etc., the M indicating one mol per liter of solution.

This work involves the use of negative exponents, concerning which the following comment may be of assistance: $10^2 = 100$; $10^1 = 10$; $10^0 = 1$; $10^{-1} = 0.1$; $10^{-2} = 0.01$; $10^{-7} = 0.000{,}000{,}1$. Such values as the last are common, but they are cumbersome and difficult to plot or chart. It is customary, therefore, to use a logarithmic derivative of the C_{H^+}, i.e., the pH, or potential of the C_{H^+}, rather than the latter itself. The pH is the logarithm of the reciprocal of the C_{H^+}, and an illustrative example of its derivation follows:

Let $C_{H^+} = 0.001\text{N}$ (or 10^{-3}, or 1.0×10^{-3})

Then, reciprocal of $C_{H^+} = \frac{1}{0.001} = 1000$

and, $\text{pH} = \log 1000 = 3.0$

Consequently, the pH increases as the C_{H^+} decreases, and vice versa. The more acid the solution, the greater the C_{H^+} and the less the pH; the less acid, the reverse will hold. Since the acidity depends upon the hydrogen ion, and the alkalinity upon the hydroxyl ion, it follows that these must be present in equivalent amounts at neutrality. H_2O may be taken as an example of a neutral solution, inasmuch as its dissociation is represented by

$$H_2O \rightleftharpoons H^+ + OH^-$$

It is a fact that the addition of H^+, as by dissolving an acid in the H_2O, will increase the $[H^+]$ and decrease the $[OH^-]$. The solution of an alkali, such as NaOH, increases the $[OH^-]$, but decreases the $[H^+]$. Neither ion, however, entirely disappears in aqueous solution, and the increase in one causes an equivalent decrease in the other. Consequently, the concentration of either indirectly measures that of the other, and, for the sake of convenience, only H^+ is considered, even alkaline solutions being described in terms of C_{H+}. The distinction, then, between acid and alkaline solutions rests upon the relative preponderance of H^+ over OH^-, or the reverse. The C_{H^+} of H_2O has been found to be 10^{-7}. In other words, a liter contains 0.000,000,1 Gm. of hydrogen in ionic form. The pH is then 7.0, and this is considered the value at neutrality—acid solutions being indicated by lower and alkaline by higher figures.

Indicators.—The color change of indicators is due to C_{H^+} or to its complement, C_{OH^-}, and for each indicator, there is a definite range of pH through which there is manifested a gradual change from the full alkaline to the full acid tint. The following list gives this range for several indicators, those italicized being the ones especially recommended for this work by Clark and Lubs.

Indicator	pH range	Color change	
		Acid	Alkaline
Thymol blue	1.2– 2.8	Red	Yellow
Töpfer's reagent (dimethylamino-azobenzol)	2.9– 4.0	Red	Yellow
Brom-phenol blue	3.0– 4.6	Yellow	Blue
Congo red	3.0– 5.0	Blue	Red
Methyl orange	3.1– 4.4	Red	Yellow
Methyl red	4.4– 6.0	Red	Yellow
Litmus (azolitmin)	4.5– 8.3	Red	Blue
Cochineal	4.8– 6.2	Yellow	Lilac
Brom-cresol purple	5.2– 6.8	Yellow	Purple
Alizarin	5.5– 6.8	Yellow	Blue
Brom-thymol blue	6.0– 7.6	Yellow	Blue
Neutral red	6.8– 8.0	Red	Orange
Phenol red (phenol-sulphonephthalein)	6.8– 8.4	Yellow	Red
Cresol red	7.2– 8.8	Yellow	Red
Thymol blue	8.0– 9.6	Yellow	Blue
Cresol phthalein	8.2– 9.8	Colorless	Red
Phenolphthalein	8.3–10.0	Colorless	Red
Thymol phthalein	9.3–10.5	Colorless	Blue

Buffers.—If acid or alkali be added to a solution of a strong base or acid, it will be found that usually the pH is markedly affected. Certain substances, however, when present in the solution, act to modify this usual effect in such manner that the change in pH may be practically inappreciable. Such substances are known as *buffers*, and they are quite common in biological fluids.

We take advantage of the properties of buffered solutions, and the color change of indicators, in the colorimetric method for determination of hydrogen-ion concentration. By mixing certain solutions in definite proportions, we can prepare mixtures of definite pH values. A suitable indicator is then chosen from the list above and added to these mixtures and to the unknown. The result is a series of graded standard tints with one of which the tint of the unknown is matched. A rough estimate of the pH of the unknown can be obtained by systematically testing it with different indicators, since reference to the list will show the pH at which their full acid or alkaline color may be expected.

The following lists give the proportions in which buffer solutions must be mixed in order to produce desired pH values. The citric acid-phosphate series was proposed by McIlvaine, but we have slightly varied the proportions in order to secure colorimetric correspondence with the buffer mixtures of Sørensen and of Clark and Lubs. The boric acid, KCl-NaOH series is that of Clark and Lubs, and the resultant mixtures in it are to be diluted to 200 cc. before use. Use $Na_2HPO_4.2H_2O$ (Sørensen's phosphate) in M/5 strength; M/10 citric acid; a solution containing 12.4048 Gm. boric acid and 14.912 Gm. KCl per liter; and M/5 NaOH.

pH	M/5 Phosphate, cc.	M/10 Citric acid, cc.	pH	Boric acid-KCl, cc.	M/5 NaOH, cc.	
2.2	0.40	19.60	7.8	50.0	2.61	
2.4	1.24	18.76	8.0	50.0	3.97	
2.6	2.18	17.82	8.2	50.0	5.90	
2.8	3.17	16.83	8.4	50.0	8.50	Dilute
3.0	4.11	15.89	8.6	50.0	12.00	each
3.2	4.94	15.06	8.8	50.0	16.30	mixture
3.4	5.70	14.30	9.0	50.0	21.30	to 200
3.6	6.23	13.77	9.2	50.0	26.70	cc.
3.8	6.77	13.23	9.4	50.0	32.00	
4.0	7.40	12.60	9.6	50.0	36.85	
4.2	7.99	12.01	9.8	50.0	40.80	
4.4	8.42	11.58	10.0	50.0	43.90	
4.6	8.82	11.18				
4.8	9.50	10.50				
5.0	9.86	10.14				
5.2	10.52	9.48				
5.4	10.94	9.06				
5.6	11.37	8.63				
5.8	11.85	8.15				
6.0	12.43	7.57				
6.2	12.82	7.18				
6.4	13.22	6.78				
6.6	14.50	5.50				
6.8	15.40	4.60				
7.0	16.47	3.53				
7.2	17.12	2.88				
7.4	17.78	2.22				
7.6	18.45	1.55				
7.8	18.95	1.05				
8.0	19.15	0.85				

The chemicals employed in the preparation of such solutions must be specially purified—a task probably not within the ability of the usual clinical laboratory. The NaOH solution must be prepared and stored as indicated on page 687. I will not give the details of this purification inasmuch as the laboratory with the equipment and experience necessary for this will have access to the literature. The buffer solutions and indicator solutions are readily purchasable, and, for the usual laboratory, I would advise that they be so obtained.

Technique.—The standard and unknown solutions are placed in test tubes for comparison. These test tubes should be of clear glass and uniform in size. They should be selected by introducing exactly 10 cc. water in each and choosing those in which it rises to the same height. It is also well to make permanent marks on the tubes at 5 and 10 cc. Enough indicator is added to secure the maximum color, exactly the same amount going into each tube, and the contents mixed. The indicator is measured with sufficient accuracy by using drops, holding the dropper in a vertical position. For the indicators that they recommend, Clark and Lubs suggest 5 drops per 10 cc., the indicator solution being 0.02% in cases of cresol red, phenol red, methyl red, and cresol phthalein, and 0.04% for the others. The indicators are used in aqueous solution, the dry dye being first dissolved by grinding in N/10 NaOH. Per 100 mg. of dye, the following volumes of alkali are employed: For cresol red, 2.88 cc.; phenol red, 3.1; methyl red, 4.07; brom-phenol blue, 1.64; brom-cresol purple, 2.78; and thymol blue, 2.38.

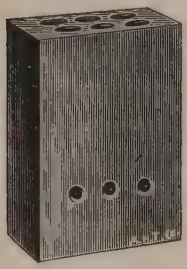

FIG. 200.—Block comparator. (*Courtesy of A. H. Thomas Co.*)

Precise determination requires many precautions as regards background, source of light, exclusion of adventitious light, etc. We have, however, found it generally satisfactory to use daylight, and to place the tubes in a rack having 3 parallel rows of 12 holes each. The back of the rack is covered with thin, plain, unglazed, white paper, which gives the background. The pH of the unknown is that of the contents of the standard tube, the tint of which is matched by that of the unknown. (See "Backing" below.)

Barnett and Chapman introduced a colorimetric system that is a great saver of labor. We have found it satisfactory, and believe it would be very generally useful, especially for isolated localities with poor laboratory facilities. Gillespie has extended its usefulness, and considers the pH values obtained accurate to about 0.1 pH at 25 to 30°C.

Place 18 test tubes in rack in 2 rows of 9 each. Beginning at the left in the front row, place 1 drop of indicator in the first tube, 2 in the second, and increase by one drop in each succeeding tube as one passes to the right. Treat the rear row similarly, but begin at the right and pass to the left. To each tube in the front row, add 1 drop (2 to 3 drops for the thymol-blue series) N/20 NaOH; to each tube in the rear row, add 1 drop N/20 HCl (use 1 drop 2% KH_2PO_4 for thymol-blue series instead of HCl). Fill all tubes to 5-cc. level with water. This standard series is viewed from the front in such a manner that the line of vision traverses 2 tubes—one in the front row and its partner in the rear row—the total amount of indicator in each pair

being 10 drops. The composite colors form a graded series, and the following table shows the pH values represented by each pair.

The block comparator shown in Fig. 200 is in common use.

Drop ratio (Front: Back)	pH values with			
	Methyl red (0.008%)	Brom-cresol purple (0.012%)	Phenol red (0.004%)	Thymol blue (0.008%)
1:9	4.05	5.3	6.75	7.85
2:8	4.4	5.7	7.1	8.2
3:7	4.6	5.9	7.3	8.4
4:6	4.8	6.1	7.5	8.6
5:5	5.0	6.3	7.7	8.8
6:4	5.2	6.5	7.9	9.0
7:3	5.4	6.7	8.1	9.2
8:2	5.6	6.9	8.3	9.4
9:1	5.95	7.2	8.65	9.75

The methyl red solution is prepared by grinding in acid-free alcohol until dissolved, and then diluting 3 volumes with 2 volumes of H_2O. The preparation of the other indicators is given above.

The unknown solution is now added to 10 drops of the indicator in a test tube until a volume of 5 cc. is reached, the solutions are mixed, and the resulting tint compared with those of the standards.

Backing.—In colorimetric comparisons, the question of "backing" is important. The unknown and standards must be balanced as regards any factors that would affect the shades of color compared. These are particularly intrinsic color or turbidity in the unknown solution, and total thickness of water columns. Moderate dilution (5 to 10 times) has no effect of moment upon the pH of many fluids, and these include most of those in which the medical man is interested. Such dilution enables one largely to discount the effect of color in the unknown. A still more accurate method of avoiding the effects of intrinsic sources of color is to place a tube of the unknown (even when diluted), without indicator, back of the standard, and one of water back of the unknown, then looking through the pair during the comparison as in the Barnett-Chapman method. Using the latter method, there should be two tubes of water back of the unknown, and a tube of the unknown without indicator back of the pair of standard tubes, thus having the line of vision traverse three tubes simultaneously. One must not rely upon a color match with the end tube of a standard series. The result should be checked by the use of a contiguous pH series. The solution being tested should be actually interposed among the standards in the rack during the comparison.

E—COLORIMETRIC DETERMINATIONS

Generally speaking, the instrument used for the comparison of colored solutions, the colorimeter, is found in two types; in one we match the intensity of the colors by varying the relative depth of the solutions, while, in the other, matching is accomplished by dilution of one solution, the depth traversed by the line of vision remaining constant. These may be called the plunger and the dilution types, respectively.

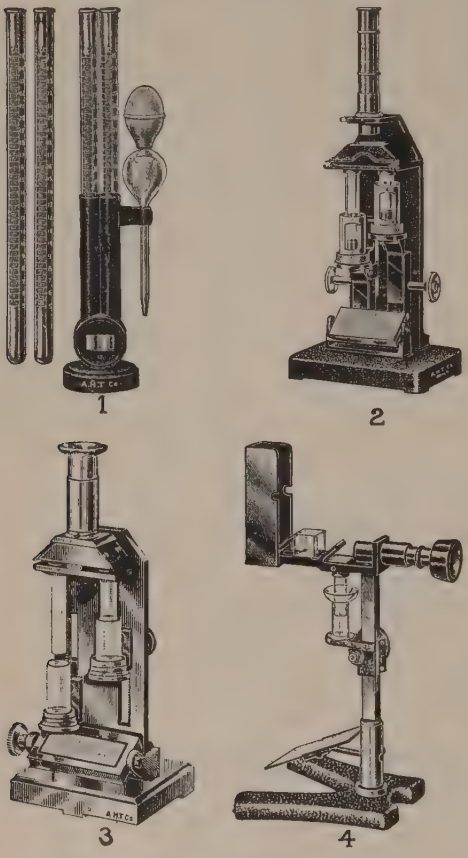

FIG. 201.

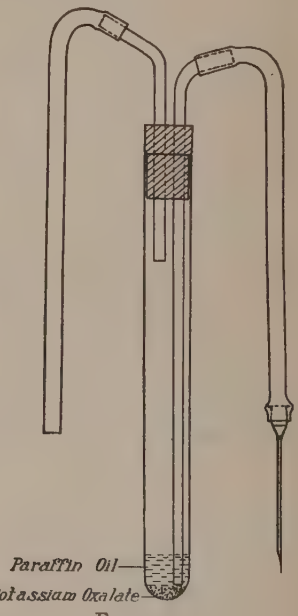

FIG. 202.

FIG. 201.—Types of colorimeter. (1) Myers; (2) Duboscq; (3) Duboscq (Pellin); (4) Bock-Benedict. (*Courtesy of A. H. Thomas Co.*)

FIG. 202.—Tube used in collecting blood. (*Journal Biological Chemistry*, 30, 289, 1917.)

With the plunger type, the relative strengths will vary inversely as the readings (depths), and the calculation follows the general formula:

$$\frac{S}{U} \times F = X$$

in which S and U are, respectively, the readings of the standard and unknown solutions when colors are matched, F is a factor, and X is the sought result. F is

constant under the conditions of each determination, and its value is determined by three considerations, viz.: (1) The actual amount (in terms of X) of substance (nitrogen, uric acid, sugar, etc.) used in preparing the standard mixture, (2) the relative volumes to which unknown and standard have been diluted, and (3) the relation of the *actual* amount of substance (blood, urine, etc.) used in preparing the unknown to the terms in which we desire to express X. For example, take the calculation for nonprotein nitrogen of blood (see p. 700). S and U are determined, and we are to express X as milligrams per 100 cc. blood.

$$\frac{S}{U} \times 0.3 \times \frac{50}{100} \times \frac{100}{0.5} = \frac{S}{U} \times 30 = \text{milligrams nonprotein nitrogen per 100 cc. blood.}$$

(1) We have used 0.3 mg. nitrogen (not ammonium sulphate); (2), standard diluted to 100 cc. and unknown to 50 cc.; therefore multiply by 50/100 (or divide by 2); (3), 5 cc. filtrate represents 0.5 cc. whole blood, and X is to be expressed in terms of 100 cc.

With the dilution type of colorimeter, the same reasoning applies, but we have here a direct proportion, and the general formula for the calculation becomes $\frac{U}{S} \times F = X$.

Permanent standards, consisting of solutions of tinted glass, are on the market for practically all examinations, but they have the faults of being subject to deterioration with age and of not affording exact tints. It is usually preferable to prepare the standard at the time of the test, using largely the same reagents as for the unknown. Standard and unknown solutions should be finished practically simultaneously. Different colorimeters are shown in Fig. 201, but one should bear in mind that Nessler tubes, such as are used in water examinations, or even test tubes, can be used quite satisfactorily for approximate results, the length of the column of fluid being measured by a ruler. In the use of a colorimeter, take precautions against retinal fatigue and adventitious light. Always check the instrument by reading the standard against itself, and, if the field is divided by a line, this line must be sharply focussed and the halves of the field made equal in area.

F—CHEMICAL EXAMINATION OF THE BLOOD

The chemical analysis of the blood has attained a clinical simplicity and significance that demand recognition for it. It provides points of value in diagnosis, prognosis, and treatment, being especially useful in nephritis, diabetes, acidosis, comatose conditions, gout, and in questions of renal function and treatment, especially dietetic. Urine findings are always dependent in part at least on the renal function, and, therefore, do not necessarily represent conditions existing in the blood. By blood chemistry, we can pass behind the barrier of the kidney.

The blood chemistry of few diseases has been as yet studied thoroughly, but our fund of knowledge is receiving constant additions. The field of tropical medicine is practically untouched, and it is quite possible that an investigation along this line might there yield facts of interest and value. The following table (amplified and altered from Myers) is a concise summary of normal findings and those encountered in various clinical conditions. The diagnostic significance is evident. Some of the

Results of Chemical Examination of Blood

Condition	Nonprotein nitrogen	Urea nitrogen	Uric acid	"Creatinine"	Sugar	Cholesterol	Chlorides	Diastase	Plasma CO_2
Normal	25–35	12–15	2–3	1–2	90–120	140–170	450–500	8–64	53–77
Exercise, short and violent	.....	.....	.....	.....	Inc.				
Exercise, long and fatiguing	.....	.....	Inc.		Dec.				
Tropics	.....	.....	.....	.....	Inc.				
Aviation (altitude)	.....	.....	.....	.....	Inc.	.....	.....	....	Dec.
Furunculosis	.....	.....	.....	.....	Inc.				
Diabetes mellitus, mild	.....	.....	.....	.....	150– 300				
Diabetes mellitus, severe	.....	to 30	4–10	2–4	300–1200	200–800	to 400	Inc.	10–50
Pancreatic disease	.....	.....	.....	.....	Inc.	.....	.....	Inc.	
Arteriosclerosis	.....	.....	Inc.	.....	Inc.	Inc.			
Kidney, passive congestion of	.....	.....	.....	.....	.....	.....	Inc.		
Nephritis, acute	.....	40–200	5–15	2–10	120–180	Inc.	450–600	....	20–45
Nephritis, interstitial, early	30–50	15–37	3–12	2–4	120–150	.....	450–600		
Nephritis, interstitial, terminal	100–350	60–300	5–27	5–28	120–300	to 300	360–600	Inc.	12–40
Nephritis, parenchymatous (nephrosis)	.....	15–60	2–5	2–4	100–200	to 900	500–610	....	to 30
Nephritis, chronic, diffuse, severe	.....	to 230	to 10	to 16	to 250	to 412			
Kidney, polycystic, double	.....	to 75	to 5	to 8.5	to 200				
Uraemia	90–350	to 300	.....	4+					
Prostatic obstruction	Inc.	to 30	3– 9	1.5–4	110–160				
Gout	.....	.....	4–10						
Hyperthyroidism	.....	Inc.	.....	.....	Inc.	to 80			
Hypoendocrine conditions	.....	Dec.	.....	.....	60–90	to 1350	Dec.	Dec.	
Pregnancy	Dec.	Dec.	.....	.....	.....	to 500			
Hyperemesis gravidarum	.....	.....	to 5						
Eclampsia	25–45	10–25	4–8	to 2.7	.....	Inc.	Inc.	....	43–58
Cholelithiasis	.....	.....	.....	.....	.....	130–300			
Acute yellow atrophy of liver	.....	.....	.....	.....	.....	to 87			
Intestinal obstruction, acute	75–170	45–170	Inc.	Inc.			Dec.		
Fever, acute	.....	Inc.	Inc.	to 4	.....	.....	Dec.		
Pneumonia, severe and late	.....	to 53	to 18	to 3.5	to 180	.....	Dec.	....	to 33
Anaemia, pernicious	to 108	to 75	to 10	to 3.1	to 300	to 60	to 600	....	Dec.
Leukaemia	.....	.....	to 10	to 2.5					
Leukaemia, myelogenous	to 110	to 20	to 7.6						
Malignancy, late	Inc.	to 58	to 10	to 3.3	.....	.....	variable	....	Dec.
Dementia precox, catatonic	.....	6–10	Dec.	.....	Inc.				
Shock	Inc.	Inc.	.....	Inc.	Inc.	.....	.....	....	Dec.
Oedema	.....	.....	.....	.....	.....	.....	Inc.		
$HgCl_2$ poisoning	to 370	to 300	to 16	to 35	120–200	to 350	to 114	....	to 25
Plumbism	Inc.	Inc.	Inc.						
Alcoholism	.....	.....	.....	.....	.....	Inc.			
Ether anaesthesia	.....	.....	.....	.....	Inc.	.....	.....	....	Dec.
Digitalis	.....	.....	.....	.....	.....	.....	Dec.		
Salicylates, antipyrin, quinine	.....	.....	.....	.....	Inc.				
Epinephrin	.....	.....	.....	.....	Inc.				

results are based upon the analysis of many cases; others upon but few. The values are given in milligrams per 100 cc. whole blood (the usual system), except those for diastatic activity (recorded in Winslow's empirical units) and acidosis (expressed in terms of plasma carbon dioxide combining power—volumes %). "Inc." and "Dec." signify increased and decreased respectively. "To," qualifying a figure, indicates that findings range from normal to that figure.

Clinical Significance.—*Interstitial nephritis* is characterized by a nitrogen retention, while *parenchymatous nephritis* has relatively little nitrogen retention but does have a decided tendency towards chloride retention. *Essential hypertonia* with its normal blood chemistry is differentiated from *arteriosclerosis* with its frequent nitrogen retention. The imminence of *uraemia* may be judged by the extent of the nitrogen retention, this finding being of aid in the differentiation of the uraemia of nephritis accompanied by a flagging heart from the passive congestion of cardiac decompensation. It is especially informative in determining which is the secondary condition, thus furnishing therapeutic indications relative to mooted questions of treatment (hot packs, morphine, renal stimulants, etc.). Unsuspected cases of nephritis showing only gastric symptoms clinically have been detected by blood chemistry. The significance of albumin in traces and occasional casts in urine has been more definitely established by examination for increase of uric acid in the blood—an increase arguing for an organic lesion. Values of over 4 for "*creatinine*" do not occur without great impairment of renal function, and findings of more than 5 have practically uniformly foretold a fatal termination in less than six months, *except in acute nephritis and mild bichloride of mercury poisoning.* The "creatinine" is also the best guide to the status of renal function in terminal cases. The chloride and nitrogen content afford guides to diet.

The surgeon must concern himself with questions of renal function, hepatic function and acidosis if he desires the best results and uneventful convalescence, and the pediatrician should always bear the possible occurrence of acidosis in mind. These conditions are considered on pp. 708, 732 and 738, and blood chemistry supplies the standard methods for their estimation.

The blood may also indicate a prediabetic state, and thus place the practitioner upon his guard. There is too a condition but recently recognized in which there is a normal blood sugar, a persistent glycuresis of usually less than 1% independent of carbohydrate intake, and occasionally polyuria, but with no other symptoms of diabetes mellitus, known as *renal diabetes*. It is apparently harmless, probably not uncommon, and may represent the condition affecting most of those "diabetics" who can disregard diet with impunity. The blood sugar and plasma CO_2 are usually considered the only safe guides in the treatment of *diabetes mellitus* and no extended medical treatment or surgical interference should ever be attempted without their estimation. Glycosuria is a poor guide, especially in advanced cases. In *comatose conditions*, nitrogen retention will indicate the uraemic, and hyperglycaemia the diabetic cases. But *acute nephritis* should always be borne in mind, as it may have a pronounced acidosis but no nitrogen retention. A high uric acid finding alone is characteristic of *gout*, and aids in differential diagnosis from simple *rheumatic fever* and other *arthritides*, any uric acid retention in them being usually accompanied by retention of other nitrogenous elements. It is especially useful in the diagnosis of gouty arthritis without tophi.

The efficacy of treatment will, in general, be shown by the degree of approach to normal blood findings.

Blood Nitrogen.—The nitrogen of the blood is contained in the proteins (serum albumin, haemoglobin, corpuscles, etc.) and is present also as a nonprotein portion. The latter is at present especially important clinically, and our chemical examination uses it, precipitating and discarding the protein portion. This nonprotein nitrogen comprises that found in the urea, uric acid, "creatinine," creatine, amino acids, and ammonia, but, ordinarily, we examine only for the first three.

Collection of sample.—We have found the Folin-Wu system of blood analysis satisfactory, and the following procedures utilize their tungstic acid filtrate and largely follow their technique. The blood is collected by the apparatus shown in Fig. 202. The oil is omitted and a small flask may be substituted for the centrifuge tube. Twenty milligrams (avoid excess) of pure powdered potassium oxalate or ten milligrams of lithium oxalate per 10 cc. blood is placed in the receptacle, the longer glass tube being used as a stirrer. For a complete examination, one should secure at least 10 cc., best taken not less than three and one-half hours after a meal, and preferably before breakfast.

The values will decrease the longer the sample stands. Twenty hours at room temperature does not change the nitrogenous substances, and the sugar only slightly. After that period, the sugar deteriorates rapidly, especially when agitated. The other ingredients show but slight change for two or three days, especially if preserved as blood filtrate and in the refrigerator.

A good routine is to examine always for urea and sugar, for CO_2 capacity if either of the preceding is abnormal, and for "creatinine" if the urea nitrogen is over 20. The nature of the findings or of the case will indicate further useful procedures.

Although there are some advantages for making a few of the estimations on plasma or serum, for clinical purposes whole blood is entirely satisfactory in the estimation of nonprotein nitrogen constituents, for sugar, chlorides and cholesterol. The use of whole blood makes for economy in time and blood, convenience and uniformity of results.

Preparation of lithium oxalate.—To 50 Gm. of lithium carbonate in a liter beaker add 85 Gm. of crystallized oxalic acid. Pour on the mixture about one liter of hot water (70°C.). Stir cautiously to avoid loss of liquid by foaming. Filter, evaporate the resulting solution to dryness, and powder.

For *precipitation of proteins*, measure 1 vol. (10 cc.) blood into a 20-vol. Erlenmeyer flask. (Bear in mind that a pipette calibrated to deliver 10 cc. of water will not also deliver 10 cc. of the more viscous blood.) Add 7 vol. H_2O by graduate, and mix. Add 1 vol. 10% sodium tungstate ($Na_2WO_4.2H_2O$), and mix. Add, slowly with shaking, 1 vol. diluted H_2SO_4. Close mouth of flask with rubber stopper, and shake about three minutes. The mixture is now of a reddish-brown color, and, upon shaking, sounds like metallic mercury and shows only traces of foam. Let stand ten to twenty minutes. Filter through dry, NH_3-free filter paper, returning the first few drops if necessary, and collect filtrate in a dry 150-cc. Erlenmeyer flask, covering funnel with a watch glass during filtration.

The tungstate should be as pure as possible, containing less than 0.5% Na_2CO_3, and its 10% solution being alkaline to phenolphthalein. But, in any case, it is best to standardize the H_2SO_4 (about ⅔ normal) against it. Pipette 5 cc. blood into

an Erlenmeyer flask; add 35 cc. H_2O and mix; rinse pipette (unless calibrated to *deliver* 5 cc. *blood*) in laked mixture; add 5 cc. tungstate and mix. Then add approximately N/1 H_2SO_4 until the criteria noted above (color, sound and foam) are obtained. Suppose 2.5 cc. acid are required. Then each 2.5 cc. must be diluted to 5 cc. (or 25 cc. to 50 cc., etc.) to secure the correct balance between tungstate and acid, and to secure the desired total volume. Foaming with a light-red color indicates not enough acid; foaming with a dark-brown color indicates too much acid. The filtrate should be neutral to Congo red. Foaming of the mixture or filtrate upon shaking indicates incomplete protein precipitation. In such a case, if the mixture is still bright-red in color, the sample may be saved by adding to it diluted H_2SO_4 drop by drop and shaking vigorously after each addition until foaming has ceased.

If, instead of whole blood, the analysis is to be made of plasma, precipitate in same manner but employ 1 vol. plasma, 5 vol. H_2O, and 2 vol. each of tungstate and acid. For corpuscles, use 1 vol. corpuscles, 8 vol. H_2O, and a half volume each of tungstate and acid.

Sugar.—Put 2 cc. blood filtrate in blood-sugar tube (see Fig. 203). In each of 2 similar tubes, place 2 cc. standard sugar solution containing respectively 0.2 and 0.4 mg. dextrose. Add 2 cc. alkaline copper solution to each tube. The surface of the mixture must now be within the constricted portion of the tube; otherwise, the tube should be discarded. Place tubes in a boiling water bath, and boil for six minutes. Transfer to cold water bath, and add 2 cc. molybdate-phosphate solution to each tube, and let stand two minutes. Dilute to 25 cc. mark (dilute unknown to match standard as closely as possible if using a tube graduated to 50 cc. (Rothberg-Evans modification)), insert rubber stopper and mix thoroughly. Compare the unknown with standards in colorimeter.

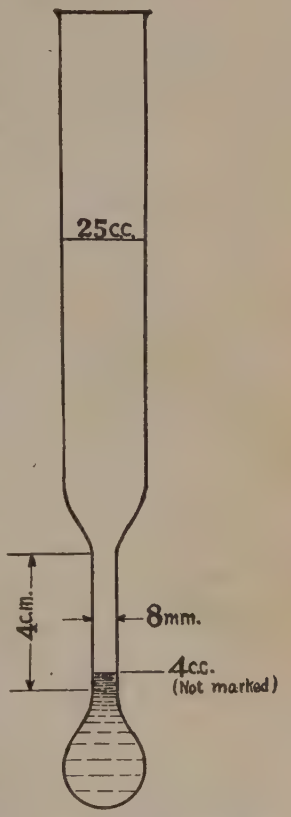

FIG. 203.—Blood sugar test tube.

Calculation: $\frac{S\ (\text{weaker})}{U} \times 100$, or $\frac{S\ (\text{stronger})}{U} \times 200$, = mg. sugar per 100 cc. of blood.

The *standard sugar solution* is prepared by dissolving 1 Gm. pure, anhydrous dextrose in 0.3% aqueous benzoic acid solution and diluting to 100 cc. with 0.3% benzoic acid solution. Benzoic acid serves as a preservative for dextrose. Mix, bottle, and stopper tightly. This stock solution keeps indefinitely. If pure dextrose is not available, a standard solution of invert sugar made from cane sugar is equally useful. Place exactly 1 Gm. cane sugar in a 100-cc. volumetric flask, add

20 cc. N/1 HCl, and let stand over night at room temperature, or rotate the flask continuously for 10 minutes in a water bath at 70°C. Add 1.68 Gm. $NaHCO_3$ and about 0.2 Gm. sodium acetate to neutralize the HCl, shake a few minutes to remove most of the CO_2, and fill to 100 cc. with H_2O. Then add 5 cc. more H_2O (1 Gm. cane sugar yields 1.05 Gm. invert sugar), and mix. *Working standard solutions* are prepared by diluting 5 and 10 cc. of the above stock to 500 cc. with 0.3% benzoic acid solution. These will contain 0.2 and 0.4 mg. dextrose per 2 cc. respectively.

Prepare *alkaline copper solution* by dissolving 40 Gm. anhydrous Na_2CO_3 in about 400 cc. H_2O, and place in liter flask. Add 7.5 Gm. tartaric acid, and, when dissolved, add 4.5 Gm. crystallized $CuSO_4$; mix, and make up to 1 liter. With impure carbonate, a sediment may form in a week or so; in such case, transfer the clear, supernatant fluid to another bottle.

For the *molybdate-phosphate solution*, put 35 Gm. molybdic acid in a 1-liter beaker, add 5 Gm. sodium tungstate, 200 cc. 10% NaOH, and 200 cc. distilled H_2O. Boil vigorously 20 to 40 minutes to remove NH_3, cool, and dilute to 350 cc. with distilled H_2O. Add 125 cc. 85% phosphoric acid, and dilute to 500 cc. with distilled H_2O.

Sugar tolerance.—The study of sugar tolerance has clinical value in a variety of conditions. The test is carried out as follows: after an overnight fast, ingest 100 Gm. dextrose dissolved in 300 cc. black coffee. Collect blood samples just before feeding and at 45 and 120 minutes after same, and determine the blood sugar in each sample. If the blood sugar has not by then returned to about normal, one may test further samples taken at hourly intervals. Venous blood not finger blood (practically arterial) should be used. The urine may also be collected and tested for sugar.

A normal person should show no glycosuria and his blood sugar will be at its maximum (about 150) within 45 minutes, and normal again after 120 minutes. The usual abnormal variation consists in increased height or, especially, prolonged duration of curve before reaching normal, as much as six hours being not unusual. The work reported on the problem of sugar tolerance indicates that it is best to interpret an abnormal curve simply as indicating an impaired sugar tolerance and its degree. High blood sugar and alimentary glycosuria may readily be produced in cases of hyperthyroidism. Hyperglycaemia may occur in nephritis due apparently to raising of the threshold point of the kidney for glucose. Hypoglycaemia and flattened sugar tolerance curves are often found in hypoendocrine conditions. Low blood sugars (60–90 mg. per 100 cc.) are reported in myxoedema, cretinism, Addison's disease and pituitary disease.

Tolerance for glucose is apparently better after carbohydrate has been given, probably due to the stimulus to the glycogen forming mechanism. To take advantage of this fact Killian has proposed the following test: a standard breakfast consisting of two slices of bread, an egg in any form and one cup of water is fed first thing in the morning. Two hours after this breakfast the patient empties the bladder and drinks 200 cc. of water. One hour after this a specimen of blood and a specimen of urine are taken to serve as controls. The patient then takes glucose 1.75 Gm. per kilo of body weight in 50% solution. Specimens of blood are now taken every hour for three or four hours, the sugar determined and a curve plotted. Following the taking of glucose a 24-hour specimen of urine is collected and glucose determined.

Nonprotein Nitrogen.—Use NH_3-free H_2O throughout. Put 5 cc. blood filtrate in a dry 25 × 250 (or 200) mm. Pyrex test tube (graduated at 35 and 50 cc.). Add

1 cc. diluted acid-digestion mixture and a dry quartz pebble, or a piece of platinum. Boil vigorously over a microburner until white, *dense* fumes form (three to seven minutes). Then lower flame until boiling is just visible, cover mouth of tube with a watch glass, and continue heating gently (at least two minutes from time of covering) until solution is clear. Remove flame, let solution cool in place for 70 to 90 seconds, and then cautiously add 15 to 25 cc. H_2O. Cool further to room temperature, and add H_2O to 35-cc. mark. Put 6 cc. working nitrogen standard solution (0.3 mg. N) in a 100-cc. volumetric flask, add 2 cc. diluted acid-digestion mixture and about 50 cc. H_2O. To the unknown, add Nessler's solution to 50-cc. mark, insert clean rubber stopper, and mix. To standard, add 30 cc. Nessler's solution, fill to mark with H_2O, and mix. Compare in colorimeter.

Calculation: $\frac{S}{U} \times 30$ = mg. nonprotein nitrogen per 100 cc. blood.

To prepare *acid-digestion mixture*, mix 300 cc. 85% phosphoric acid with 100 cc. concentrated H_2SO_4 (nitrogen-free) and 50 cc. 5% $CuSO_4$, in a tall cylinder. Cover well to exclude NH_3, and set aside for sedimentation of $CaSO_4$. In the course of a week or so, the top part is cleared, and 50 to 100 cc. can be removed with a pipette. Dilute the clear acid with an equal volume of H_2O for use in the method.

To prepare *stock Nessler's solution*, put 150 Gm. KI and 110 Gm. iodine in a 500-cc. Florence flask, add 100 cc. H_2O and an excess of metallic Hg (about 160 Gm.), and shake flask continuously and vigorously for 7 to 15 minutes, or until the dissolved iodine has nearly disappeared. When the red solution is visibly paler, although still red, cool it in running water and continue shaking until the reddish color of the iodine has been replaced by the green of the double iodide. This whole operation does not usually take more than 15 minutes. Now separate the solution from the surplus Hg by decantation, and wash with liberal quantities of distilled H_2O. Dilute solution and washings to 2 liters. The *working Nessler's reagent* is made by adding 75 cc. of the stock and 75 cc. distilled H_2O to 350 cc. 10% NaOH; let stand three days in tall cylinder and use the clear, supernatant fluid. To secure the *necessary crystal-clear* solutions for colorimetric comparison in nitrogen determinations, this Nessler's solution must be added after the solution to be tested has been diluted as much as practicable. Add it slowly and during constant twirling, letting it fall into the vortex of the whirlpool formed.

Sodium hydroxide solution should be made from a saturated solution of sodium hydroxide which has stood for some time allowing the carbonates to settle out. Titrate the 10% solution against N/10 hydrochloric acid and adjust. 1 cc. of 10% sodium hydroxide requires 25 cc. of N/10 hydrochloric acid for neutralization.

The *stock nitrogen standard solution* contains 4.716 Gm. $(NH_4)_2SO_4$ (1 Gm. N) per liter. For the *working standard* (10 cc. contains 0.5 mg. N), dilute 5 cc. to 100 cc. The purest salt obtainable must be used, and this is best prepared by passing NH_3 gas (heat Aq. ammon. fortior, U. S. P.) into 30% (by volume) H_2SO_4 (purest obtainable) until alkaline to litmus. Evaporate in large beaker to dryness on water bath. Dissolve in smallest possible volume of hot H_2O, and precipitate with 3 vol. 95% alcohol. Decant clear, supernatant fluid, and dry the precipitate on water bath. Repeat two more times, and finally dry for three to four hours at 110°C. Store in desiccator.

To prepare *ammonia-free water*, add a little dilute H_2SO_4 to distilled water, and distil. Select such portions of filtrate as show no color reaction with Nessler's solution. The water will absorb NH_3 from the laboratory air, and it is best to distil elsewhere, and further to protect the product by some means such as that used for preserving CO_2-free H_2O (p. 687), using in this case a guard tube of dilute H_2SO_4 instead of sodalime.

Urea.—Use NH_3-free H_2O (see above) throughout. Put 5 cc. blood filtrate in a clean, dry test tube of 75-cc. capacity. A tube that has contained Nessler's solution will probably prevent urease action, and must be cleaned thoroughly with HNO_3 before use. Add 2 drops pyrophosphate mixture (14 Gm. sodium pyrophosphate, 2 Gm. 85% phosphoric acid, and H_2O to make 100 cc.), 1 cc. urease solution, and heat in water at 40°C. for 10 minutes. Aspirate NH_3 into 2 cc. N/20 HCl diluted with about 10 cc. of H_2O by means of apparatus shown in Fig. 10. Two cubic centimeters 10% NaOH and a drop of paraffin oil, or 5 to 10 drops amyl alcohol, are added to contents of test tube, which is then quickly placed in middle of series shown and stoppered. The air current is slow for two to three minutes, and is then gradually increased as much as possible. Twenty minutes is sufficient, and the tip in the acid is then rinsed with H_2O. Dilute contents of acid tube to about 20 cc. Rubber connections must be perfectly clean, and it is preferable to employ specially perforated tips in both acid tubes.

Put 6 cc. working N standard (0.3 mg. N) in a 100-cc. volumetric flask, and fill two-thirds full with H_2O.

To unknown add 2.5 cc. Nessler's solution, fill to 25 cc. with H_2O, and mix. To standard, add 10 cc. Nessler's solution, fill to mark, and mix. Compare in colorimeter.

$$\text{Calculation: } \frac{S}{U} \times 15 = \text{mg. urea } \textit{nitrogen} \text{ per 100 cc. blood.}$$

We have found another method clinically satisfactory, and especially convenient for isolated determinations. Pipette 1 cc. urease solution into a large test tube, add 2 to 3 drops pyrophosphate mixture and 2 cc. oxalated blood, and heat in water bath at 40°C. for 10 minutes. Remove, add 13 cc. H_2O, and mix; add 2 cc. 10% sodium tungstate, and mix; and, finally, add 2 cc. adjusted H_2SO_4 (as for protein precipitation). Shake vigorously, let stand 10 to 20 minutes, and filter, returning filtrate to filter until it runs clear and colorless. Dilute 5 cc. filtrate to about 20 cc. with H_2O. Put 3 cc. working N standard (0.15 mg. N; see p. 701) in a 50-cc. volumetric flask, and dilute to about 35 cc. Add 3 cc. Nessler's solution (see p. 701) to unknown and 6 cc. to standard; make former up to 25 cc. and latter to mark with H_2O; mix, and compare in colorimeter. With low and normal blood urea, the color of the unknown often matches poorly.

$$\text{Calculation: } \frac{S}{U} \times 15 = \text{mg. urea } \textit{nitrogen} \text{ per 100 cc. blood.}$$

Urease solution is prepared by transferring about 3 Gm. permutit to a 200-cc. flask or bottle. Wash well by decantation, once with 2% acetic acid, and then twice with H_2O. Add 100 cc. 30% alcohol to moist permutit in flask, introduce 5 Gm. jack-bean meal, and shake 10 minutes. Filter, and collect filtrate in 3 or 4 small, clean bottles. At ordinary room temperature, it will remain serviceable for

at least one week if not exposed to direct sunlight, and, on ice, for three to five weeks. *Shake before using.*

Hench and Aldrich, and Schmitz, have shown that the concentration of the combined nitrogen from the urea and NH_3 in the saliva averages approximately 90% of the blood urea nitrogen, and can be utilized clinically in case it is not practicable to determine the latter. We have found the following technique suitable. Brush the teeth carefully, and rinse the mouth well with water. Chew paraffin, and collect about 25 cc. of saliva. Filter through several layers of gauze. Titrate a portion, using methyl red as the indicator, and neutralize 5 cc. on the basis of the titration. To this neutralized saliva in a test tube, add 1 cc. urease solution and 2 drops pyrophosphate mixture. Heat in water bath at 40°C. for 10 minutes. Transfer quantitatively to a 200-cc. volumetric flask containing about 2 Gm. permutit, using not more than 10 cc. H_2O. Agitate gently but continuously for five minutes, and then proceed as for ammonia in urine at the same stage (p. 724).

Calculation: $\frac{S}{U} \times 10$ = mg. N from urea and NH_3 per 100 cc. saliva.

"Creatinine."—Put 10 cc. blood filtrate in a small flask or test tube, and 5 cc. working creatinine standard in another flask. Dilute standard with 15 cc. H_2O. Add 5 cc. freshly prepared alkaline picrate (15 cc. saturated solution of *pure* picric acid mixed with 3 cc. 10% NaOH) to the filtrate and 10 cc. to the standard, and mix. Compare in colorimeter after standing 10 minutes and before 15 minutes have elapsed.

Calculation: $\frac{S}{U} \times 1.5$ = mg. "creatinine" per 100 cc. blood.

Prepare *stock creatinine standard* by dissolving 1 Gm. creatinine (or 1.6106 Gm. creatinine-zinc chloride) in sufficient N/10 HCl to make 1 liter. Place 6 cc. of the stock in a liter volumetric flask, add 10 cc. N/1 HCl, dilute to mark with H_2O, and mix. Transfer this *working standard* (5 cc. contains 0.03 mg. creatinine) to bottles, and add 4 to 5 drops xylene.

Uric Acid (Folin).—Place 5 cc. blood filtrate in 15 cc. graduated centrifuge tube. Add 7 cc. silver lactate solution. Centrifuge. Decant and discard supernatant liquid as completely as possible (by quickly inverting tube). Add 1 cc. acid sodium chloride solution (10% NaCl in N/10 HCl) and stir with a fine glass rod. Add 4 cc. water and stir again. Centrifuge. Decant supernatant liquid into a 25 × 150 mm. test tube graduated at 25 cc. Place 5 cc. uric acid working standard in a similar tube. Add to each tube: *exactly* 1 cc. uric acid reagent, 2 cc. water, exactly and with gentle shaking 2 cc. cyanide solution (15% NaCN in N/10 NaOH) *deadly poison.* Let stand at room temperature for two minutes. Transfer to boiling water bath and heat 70 to 80 seconds. Cool, fill to mark with water and compare in colorimeter.

Stock uric acid standard.—Transfer 200 mg. of pure uric acid to a clean beaker Transfer with a cylinder 20 cc. 0.5% lithium carbonate solution to another beaker; dilute with 10 cc. water, heat to 60 or 70°C., and pour this warm alkali on the uric acid and stir until the uric acid is completely dissolved. As soon as complete solution is obtained, add 10 cc. 40% formaldehyde and then 2 cc. 50% acetic acid. Transfer quantitatively to a 200 cc. volumetric flask, dilute to volume, mix, and

transfer to a series of small glass stoppered bottles filling each completely. This solution contains 1 mg. uric acid per cc. and keeps well. Store in a dark place.

Uric acid working standard.—Transfer 1 cc. stock standard, containing 1 mg. uric acid, to a 250 cc. volumetric flask. Half fill the flask with water; then add 10 cc. normal two-thirds sulphuric acid, and 1 cc. (but no more) of 40% formaldehyde. Dilute to the mark with water and mix. This solution keeps for at least a month. 5 cc. contains 0.02 mg. uric acid.

Uric acid reagent (Folin and Trimble).—Transfer 50 cc. 85% phosphoric acid and 160 cc. water to a 500 cc. pyrex flask. Heat nearly to boiling and add 100 Gm. sodium tungstate. The mixture begins to boil from the heat of the reaction. Boil gently but continuously over a micro burner for one hour, using a 10 cm. funnel in which a 200 cc. florence flask filled with cold water is placed, as a condenser. Transfer 25 Gm. lithium carbonate to a liter beaker. Add 50 cc. 85% phosphoric acid and 200 cc. water. Boil off the carbon dioxide and cool. Mix the two solutions and dilute to one liter.

Silver lactate 5% in lactic acid 5%.—Dissolve 22.5 Gm. silver nitrate in about 200 cc. distilled water in a liter florence flask. Add an excess (about 60 cc.) of 20% sodium or potassium hydroxide, add distilled water to about one liter and shake. Allow to stand for a few minutes and decant the supernatant fluid. Add distilled water to about one liter and shake, allow to stand for a moment and decant the supernatant fluid. Repeat this washing by decantation until the wash water fails to react alkaline to litmus. The total washing process need not take longer than 10 minutes. After the last decantation make up to a volume of about 200 cc. with distilled water and add 35 cc. lactic acid (Sp. Gr. 1.2) and 25 cc. 10% sodium hydroxide. Shake thoroughly and dilute to 500 cc. with distilled water. Filter through a dry folded filter, returning the first portions until the filtrate appears as clear and colorless as distilled water. Filter the entire portion through this filter. Protect from sunlight during preparation and preserve in amber glass stoppered bottles.

Chlorides.—Whitehorn's method utilizes the tungstic acid filtrate from either whole blood or plasma, thus determining chlorides of whole blood or plasma. The reagents must, of course, be halogen-free. Put 10 cc. blood filtrate and 5 cc. $AgNO_3$ solution (4.791 Gm. per liter: 1 cc. equals 1.65 mg. NaCl) in a porcelain dish, stir thoroughly, add about 5 cc. U. S. P. HNO_3, and let stand five minutes. Add about 0.3 Gm. powdered ferric ammonium sulphate, and titrate with NH_4CNS or KCNS solution of which 5 cc. balances 5 cc. of $AgNO_3$ solution, to a salmon-red color that persists for at least 15 seconds despite stirring. Calculation: 165 (5 minus cc. of KCNS sol. required) = mg. chlorides (as NaCl) per 100 cc. blood.

Cholesterol (Sackett's modification of Bloor's method).—Put 9 cc. ethyl alcohol in a 15 cc. graduated centrifuge tube. Add 3 cc. ether, and mix by inverting. Drop in slowly 0.2 cc. whole blood or plasma. Add a pinch of Lloyd's alkaloid reagent (to remove interfering color). Cork tightly with a clean cork stopper and shake vigorously for about 1 minute. Let lie horizontally with the sediment evenly distributed along the tube for 30 minutes. Centrifuge rapidly for three minutes and decant supernatant fluid completely into a small beaker. Evaporate just to dryness on a water bath. Extract the cholesterol twice, for about 2 minutes each time, with small portions (2 to 2.5 cc.) of choloroform and decant into a 10 cc. graduated cylinder. Let cool and then make up to 5 cc. mark. Measure 5 cc. of a standard

cholesterol solution in chloroform (containing 0.4 mg. of cholesterol) into a similar 10 cc. cylinder. To each of the solutions add 2 cc. of acetic anhydride and 0.1 cc. of concentrated sulphuric acid. Mix by inverting several times and then set away in the dark for 15 minutes. Transfer immediately to the cups of the colorimeter and compare as usual, setting the standard at 12 or 15 mm.

Calculations.—$\frac{\text{Reading of Standard}}{\text{Reading Unknown}} \times 200$ equals milligrams of cholesterol per 100 cc. of whole blood.

Alcohol should be pure 95%. *Ether* should be of purity equal to that used for anesthesia. *Chloroform* should be kept over calcium chloride and filtered before use.

Stock standard contains 0.2 Gm. cholesterol in 200 cc. of chloroform.

Working standard is made by diluting 8 cc. of stock standard to 100 cc. with chloroform (5 cc. contains 0.4 mg. cholesterol).

Calcium and Phosphorus.—The metabolism of calcium is so intimately connected with that of phosphorus that the one may not be considered apart from the other, either from the standpoint of the needs of the organism or in the study of bone growth and repair. Other factors involved are the parathyroids, fat soluble vitamin D and ultra violet light (sunlight) (pp. 770 and 778).

During the period of bone growth in the healthy child the normal inorganic phosphorus content averages 5 to 5.5 mg. per 100 cc. serum. While in the normal well nourished adult the average is 3.7 mg.

In adults and children the normal serum calcium is 9 to 11 mg. per 100 cc. Following major fractures in the adult the inorganic phosphorus commonly rises to between 4 and 5 mg. per 100 cc. serum, the rise taking two to four weeks and falls to normal following repair of the fracture. As a rule cases with non-union do not show this reaction in the inorganic phosphorus following fracture.

	Milligrams Per 100 cc. Serum			
	Calcium		Inorganic phosphorus	
	Adult	Child	Adult	Child
Normal	9–11	9–11	3.7	5–5.5
Major fracture adult	Slight Inc.	.....	to 4 or 5	
Tetany	.....	7.0	.....	Decrease when associated with rickets
Rickets	.....	Dec.	.....	3.7 to 2.0
Terminal nephritis (with retention acidosis)	to 5.0	.....	to 15.0	

In active rickets the inorganic phosphorus of the serum is usually found to be reduced to 3.5 mg. or lower. Although this finding cannot be considered as pathognomonic of rickets, it may be a valuable aid in the early diagnosis of such a condition.

If the concentration of calcium expressed in mg. per 100 cc. be multiplied by the concentration of inorganic phosphorus expressed in the same terms we obtain a product which in the normal child is between 50 and 60. When the product is below 30 rickets is invariably present (Howland). With products between 30 and 40 rickets is usually present. In cases which give higher products rickets is not present or repair is taking place. In infantile tetany and parathyroprivic tetany blood calcium is always diminished (usually below 7.0) but seems to bear no relation to the severity of the tetany. Tetany appears to be the only condition other than terminal nephritis with retention acidosis which shows a marked change in serum calcium (Myers). These cases of nephritic acidosis may also show extremely high figures for inorganic serum phosphorus.

Calcium (Tisdall).—To 2 cc. of serum in a 15 cc. graduated centrifuge tube add 2 cc. distilled water and 1 cc. of ammonium oxalate solution. The outside diameter of the tube should be 6 to 7 mm. at the 0.1 cc. mark. The contents of the tube are then thoroughly mixed by holding the upper end of the tube and tapping the lower end with the finger giving it a circular motion. Allow to stand for 30 minutes or longer and again thoroughly mix. Centrifuge until the precipitate is well packed. Decant, keep inverted and drain on a filter paper for five minutes. Wipe the mouth of the tube with a cloth to remove remaining moisture. Wash the sides of the tube with 3 cc. of ammonia water (2%). Stir up the precipitate by tapping as above. Recentrifuge and drain as described. Add 2 cc. of N/1 sulphuric acid, blown on so as to facilitate suspension. Place in boiling water bath for one minute. Titrate the oxalic acid, (liberated from the calcium oxalate precipitated from serum) with N/100 $KMnO_4$, using a micro-burette, to a definite pink color. Titrate at not less than 70°C. To obtain a true reading the amount of N/100 $KMnO_4$ necessary to give the faint but distinct pink color to an equal volume of water should be subtracted from the amount of N/100 $KMnO_4$ used in the titration.

Calculation: 1 cc. N/100 $KMnO_4$ = 0.64 mg. calcium oxalate or 0.20 mg. calcium.

$$X = \text{number cc. of N/100 } KMnO_4 \text{ used.}$$
$$10X = \text{milligrams calcium per 100 cc. serum.}$$

A blank determination should be run with reagents to be sure that all are calcium free

Reagents: 1. Ammonium oxalate (calcium free) 4% solution.

2. Ammonia water, 2% solution in wash bottle.

3. N/1 sulphuric acid.

4. Sulphuric acid 50% solution (by volume).

5. N/10 oxalic acid in N/10 sulphuric acid (for use in titration of N/100 $KMnO_4$).

6. N/100 $KMnO_4$ solution: make from N/10 $KMnO_4$ solution (3.161 Gm. to one liter) and standardize against N/10 oxalic acid daily. Method of titration: to 1 cc. of N/10 oxalic acid in a beaker add 25 cc. hot water and 5 cc. 50% sulphuric acid. Place on water bath and carry out titration, with constant stirring,

at not less than 70°C. 10 cc. N/100 $KMnO_4$ is equivalent to 1 cc. of N/10 oxalic acid.

Inorganic Phosphorus in serum (Benedict-Theiss).—Place 2 cc. of clear, non-haemolized serum in a test tube, add 4 cc. distilled water and 4 cc. 20% trichloracetic acid. Stopper and shake briskly until complete precipitation has been effected. Allow to stand for at least 10 minutes and filter through a dry ashless filter. Place 5 cc. of the filtrate in a Rothberg-Evans sugar tube. In a similar tube place 5 cc. of phosphorus standard (containing 0.025 mg. phosphorus). Now add to *each tube:* 3 cc. sulphuric acid 16%, 1 cc. molybdic acid reagent which has been diluted 1 to 1 with distilled water, and 1 cc. of the hydroquinone bisulphite reagent. Mix, stopper loosely and place in a boiling water bath for 10 minutes. Cool, dilute if necessary, and compare in colorimeter.

Calculation: $\frac{S}{U} \times 2.5$ = milligrams of inorganic phosphorus per 100 cc. serum.

Sulphuric acid 16% (by weight).

Molybdic acid reagent.—To 20 Gm. of pure molybdic acid in a beaker add 25 cc. of 20% sodium hydroxide and warm gently until the molybdic acid dissolves. Cool and dilute to 250 cc. with distilled water. Filter if necessary through an ammonia free filter paper.

Hydroquinone bisulphite reagent.—Dissolve 15 Gm. of sodium bisulphite and 0.5 Gm. of hydroquinone in sufficient distilled water to make 100 cc.

Phosphorus standard stock.—Dissolve 0.4386 Gm. of anhydrous acid potassium phosphate (KH_2PO_4) in 500 cc. of distilled water in a liter flask and make up to the volumetric mark with distilled water. Preserve with chloroform. 1 cc. of this solution contains 0.1 milligram of phosphorus.

Phosphorus standard, working.—Place 5 cc. of stock standard in a 100 cc. volumetric flask and dilute to mark with distilled water. Preserve with chloroform. 5 cc. contains 0.025 milligram phosphorus.

Oxygen.—The standard method for determination of *haemoglobin* in blood is the measurement of the *oxygen capacity.*

One can employ the blood-gas apparatus of Van Slyke (see p. 714). Wash apparatus twice with water, and then introduce 6 cc. water, 0.3 cc. 1% saponin (Merck), and 2–3 drops caprylic alcohol. Evacuate and shake for 15 seconds to extract air, and repeat until reading is constant. Force about 6 cc. back into cup. Place 3 or more cubic centimeters oxalated, carbon monoxide-free blood in a separatory funnel, and rotate in a thin layer for a few minutes. Open upper cock of apparatus slightly, so that fluid flows inside slowly, stir blood thoroughly, and, with the tip of the pipette reaching the bottom of the cup, run 2 cc. blood into the burette, slowly so that less than 2 mm. is in cup at any time. Add 0.1–0.12 cc. 20% potassium ferricyanide, rendered air-free by boiling, and then cooled, before last cubic centimeter of fluid in cup has been admitted. Seal capillary above upper cock with mercury, evacuate, and shake for 3 minutes. Release vacuum, and then introduce slowly under *slight* negative pressure 0.5 cc. N/2 NaOH, which has previously been saturated with air or oxygen, and then a little Hg. When drainage of alkali is complete, read the volume of the gas.

Calculation is based on Haldane's scale, which considers 18.5 vol. % oxygen as representing the clinical normal, or 100% haemoglobin: Multiply the gas volume

by the proper factor in the second column of Table, page 717; subtract 2.1 to give oxygen capacity; multiply this result by 5.41 for clinical percentage of haemoglobin, or by 0.746 for percentage of haemoglobin by weight.

One can collect the blood under paraffin oil (see p. 714), and determine the *oxygen content* by the same technique. Here, the blood is transferred directly to the apparatus without saturation with air. After multiplying gas volume by factor in second column of Table, page 717, for (*a*) oxygen content, subtract 1.36 vol. %; (*b*) oxygen bound by haemoglobin in venous blood, subtract 1.5; (*c*) oxygen bound by haemoglobin in arterial blood, subtract 1.7; and the % of the total haemoglobin saturated with oxygen, would be $\frac{100b}{O_2 \text{ capacity}}$, or $\frac{100c}{O_2 \text{ capacity}}$; and, vol. % *oxygen unsaturation*, would be O_2 capacity minus b, or O_2 capacity minus c.

Acidosis

Everyone is familiar with that form of respiratory disturbance associated with diabetic coma that is known as Kussmaul's air hunger. Here we have hyperpnoea, a form of dyspnoea typically without cyanosis, and furnishing the best clinical evidence of acidosis. Acidosis, however, is now recognized to be but a particular phase of disturbance of the *acid-base equilibrium* of the body, and our conceptions of its features and its intricate relationships have radically changed.

Van Slyke restricts the use of the term "acidosis" to describe a condition caused by acid retention sufficient to lower either the bicarbonate or the pH of the blood below normal limits. The pH (see p. 687) of the blood may be considered the danger sentinel; as long as it is normal, the acid-base equilibrium is normal or compensated; otherwise, it is uncompensated, and life is seriously threatened. The normal pH of the blood may be given as 7.3 to 7.5 (a slightly alkaline reaction), each individual, however, probably having normally narrower limits of variation. That of the blood serum is about 0.2 pH higher, and that of the other body fluids (not the excretions) probably closely approximates and promptly follows any change in that of the blood plasma. Variations to the acid side may, for a short time at least, be as low as 7.0, although not much lower without fatal results; 7.0 is considered the point where coma occurs. Variations to the alkaline side (*alkalosis*) beyond 7.8 are accompanied by symptoms of tetany, although one is not at present justified in assuming that all tetany is either caused, or accompanied, by alkalosis. In fact, tetany can occur without alkalosis, and, instead of the latter, the real exciting cause is believed by some to be diminished O_2 supply to the tissues, or some disturbance of ionic equilibrium, such as relative increase of Na ion or decrease of Ca ion or Cl ion. So, the extreme range of reaction compatible with life probably lies approximately between pH of 7.0 and 7.8.

The severe reactions following intravenous medication or infusions may be due, at least in part, to the fact that the pH of the fluid introduced is decidedly more acid or alkaline than that of the blood. This applies to solutions of glucose, the salines, and possibly also to sodium citrate, arsphenamine, sera, antitoxins, etc. The reaction of such solutions should be carefully investigated as prepared or before injection and properly buffered with suitable phosphate mixtures if necessary.

The hydrogen-ion concentration (or its derivative, pH) of the blood varies as the ratio between the concentrations of dissolved carbonic acid and bicarbonate, generally indicated by $\frac{[H_2CO_3]}{[NaHCO_3]}$, i.e., a relative increase in the H_2CO_3 increases the hydrogen-ion concentration and lowers the pH, and vice versa. The stability of this ratio is preserved by body mechanisms operative in controlling its two factors—the H_2CO_3 being under respiratory control, and the $NaHCO_3$, considered as representing the alkali reserve, being normally maintained by food.

The erythrocytes regulate the concentration of bicarbonate by virtue of their haemoglobin and the reversible reaction.

$$H_2CO_3 + NaCl \rightleftharpoons HCl + NaHCO_3$$

Other acids are transported in the red cells in the same manner, being excreted mostly by the kidneys. The HCl and some H_2CO_3 pass into the cell, and are held by the haemoglobin and phosphates, the haemoglobin transporting in this fashion 75 to 80% of the CO_2. In the lungs, the CO_2 is excreted and NaCl reformed. This ability of haemoglobin to form bicarbonate is important inasmuch as the corpuscles can conceal 5 to 10 times as much acid as the plasma bicarbonate can ordinarily neutralize. A full appreciation of the significance of this ratio being the basis of an intelligent comprehension of acid-base equilibrium, a detailed analysis of factors that tend to influence the ratio is given.

Factors operating:

A. To increase or protect bicarbonates:

1. Administration of bicarbonate.
2. Loss of gastric HCl induced by obstructing the pylorus, and regularly washing out the stomach for some days.
3. Processes indicated by increased excretion in the urine of ammonia compounds, the ammonia being probably diverted from urea formation, and of substances producing a titrable acidity, they including buffer acids, such as acid phosphates.
4. Possibly a shift of HCl to the tissue cells from the plasma like that from the plasma to blood cells.

B. To decrease bicarbonate:

5. Acid substances, by their
   (*a*) Increased production.
   (*b*) Decreased elimination, or
   (*c*) Ingestion.
6. Diuresis, with elimination via the urine.
7. Lack of Factor A (3).
8. The hyperpnoea associated with deficient oxygen.

C. To increase carbonic acid:

9. Administration of carbonic acid.
10. Impaired diffusion in the alveoli of the lungs.
11. Slowing of respiration.

D. To decrease carbonic acid:

12. Hyperpnoea.
    (*a*) Voluntary.
    (*b*) Due to disease processes.
    (*c*) Due to low oxygen content of air.
    (*d*) Emergence from warm water.
13. Low atmospheric content of CO_2.

Figure 204 is a graphic representation of essential facts in acid-base equilibrium. Ordinates represent total CO_2 (or CO_2 content, which comprises that in simple solution and that as bicarbonate) of *whole blood* in volumes per cent, and abscissae the mm. CO_2 tension in the blood as drawn. The line OT gives the proportion of total CO_2 present in simple solution. pH values are shown by the lines OL, OM, etc.

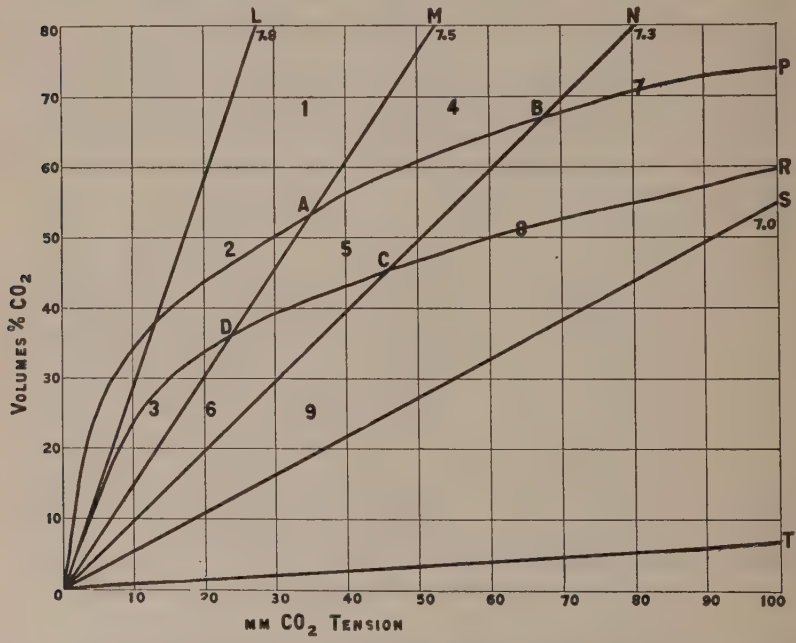

FIG. 204.—Carbon dioxide absorption curves. (Modified from Peters, Barr, and Rule, and Van Slyke.)

The extreme normals for carbon-dioxide absorption curves are OP and OR. The CO_2 tension of alveolar air may be the same or vary as much as 20 mm. below, while that of venous blood will be about 6 (0.8–10.0) mm. higher than that of arterial blood. The "CO_2 capacity" (or "CO_2 combining power") of plasma may be as much as 15 vol. % more than the total CO_2 of whole blood.

The actual state of acid-base balance, then, can only be determined by the use of any two of a number of interdependent variables, such as total CO_2, CO_2 tension,

pH, H_2CO_3 concentration, other buffers than bicarbonate, plasma chloride, ratio of oxyhaemoglobin to haemoglobin, etc. Findings that fall within ABCD and at about 40 mm. tension indicate a normal equilibrium for the resting individual at ordinary altitudes. Or, such a normal would be a total CO_2 of about 49 (43–56) vol. % for whole blood, and 50–65 vol. % for plasma. The normal for the individual falls within narrower limits.

If either H_2CO_3 or bicarbonate varies from normal values, there is apparently an effort on the part of the body to compensate by adjusting the other so as at least to maintain a normal pH. This is accomplished by respiration, or by diverting alkali from or recalling it to the blood stream. Naturally, treatment of any such abnormal condition will do well to imitate Nature's efforts. Haggard and Henderson have demonstrated that blood alkali may be decreased in two ways—by acids (the *acidotic process*) or by acapnia (the *acapnial process*). By the forced breathing of the acapnial process the lungs are over-ventilated and an excessive amount of carbon dioxide is washed out of the blood, thus bringing on a temporary alkalosis. In case of prolonged forced breathing, nature prevents an extreme alkalosis by causing the alkali to leave the blood, it being stored in the tissues or excreted in the urine. Blood relatively poor in carbonic acid or relatively rich in alkali acts to depress respiration, and the slowing of respiration produces an acidosis by the resultant retention of H_2CO_3, this causing alkali to be recalled from the tissues. Thus acidosis, in calling more alkali into the blood from the tissues, represents what may be regarded, in this case, as a restorative effort. Hence, administration of bicarbonate is indicated in acidotic processes, and of CO_2 in acapnial; the use of the wrong one is dangerous.

The numbered regions of the chart are associated with various clinical conditions, e.g., tetany from 1, 2, and 3; the acidosis of diabetes mellitus, nephritis, or infantile marasmus with 6 or 9; pneumonia, morphine narcosis, and breathing of air containing 3–5% CO_2 with 7 or 8; emphysema with 4; some cardiac cases with 9; overdose of bicarbonate with 1 or 4; fever with 2; as the result of high altitudes, 2 or 3, or, when acclimated, 6; shock (handling of intestines), deep ether anaesthesia, and carbon monoxide asphyxia with lowered bicarbonate. The disturbance of acid-base equilibrium in the last is the result of acapnia.

Normal metabolism results in the constant formation of acids, especially H_2CO_3, and disease processes may occasion the presence of still more. A constant loss of alkali results, the neutralization products being eliminated mostly in the urine, and the H_2CO_3 via the lungs; the body fluids are excellently buffered (see p. 690), the most important buffers being bicarbonate, proteins (especially haemoglobin) and phosphates. In the maintainance of the normal pH, the CO_2 (or H_2CO_3) is the easily variable factor. The onslaught of invading acids is first met by the bicarbonates (acidotic process); hyperpnoea lowers the H_2CO_3, and a normal pH is maintained until the bicarbonates are reduced to one-fourth (perhaps even to one-eighth) of their normal concentration. If, nevertheless, the pH falls (and only then), the other buffers are used, and, if it reaches 7.0, most of the remaining bicarbonate becomes available. The blood handles the situation, but buffers from the tissues or other body fluids also become available in extreme cases.

Acidotic acidosis is due to either the abnormal formation or ingestion of acid substances, or to decreased elimination of normal metabolic products. Ketosis is an

important example of the former, and retention of acid phosphates of the latter. In either case, the body is robbed of its bases. The appearance of an acidosis in disease constitutes a serious complication demanding immediate attention. Acidosis is usually present at time of death and may be its immediate cause. We must be prepared for it in terminal malignancy, severe pneumonia, wood alcohol poisoning, starvation (whether the result of ulcer, malignancy, or other causes), diabetes mellitus, any form of nephritis, severe eclampsia, salicylate poisoning, following chloroform and ether anaesthesia (especially in females), cholera, yellow fever, blackwater fever, kala-azar and heat stroke. In infants and children, it is especially important, and may occur alone, or as the result of improper diet, or be a feature of alimentary tract disturbance (diarrhoeas, cyclical vomiting, etc.). The acidosis of diabetes mellitus is characterized by ketosis and increased NH_3 quotient; while that of nephritis is a phosphate retention without ketosis, and, as one would expect, the NH_3 quotient is usually not increased. Infantile diarrhoea with ileocolitis shows a marked ketosis; but, lacking the ileocolitis, the ketosis is only moderate, and the acidosis is due to phosphate retention.

The *surgeon* is particularly concerned with the influence of anaesthesia upon acid-base equilibrium, ether causing a decrease of 4–17 vol. % in plasma CO_2 capacity, and $CHCl_3$ having an even more pronounced effect. In spinal or gas-oxygen anaesthesia, the fall is but slight. Haemorrhage and shock enhance the danger. So, evidence of even slight acidosis should be corrected by administration of bicarbonate prior to operation.

Relative to the *administration of bicarbonate* in treatment, there is now a decided reaction against the use of amounts that may prove injurious by reason of the danger of tetany. There is a tendency to employ it only in decompensated acidosis, and control it by estimations of plasma CO_2 capacity. 0.5 Gm. $NaHCO_3$ per 19 kg. body weight will raise the plasma CO_2 capacity by 1 vol. %. It is distinctly contraindicated in cases whose low plasma CO_2 is due to acapnial processes. The brilliant results of Sellards in cholera directed attention to its usefulness in nephritis generally. It is of only minor value in diabetes mellitus, and then only in long and stubborn or severe acidosis. Early administration, is desirable in children, and good results are obtained, especially with the older ones. An acidosis, however, once established in infants may cause death despite alkali. In order to avoid over-dosage of bicarbonate, methyl red, which is more sensitive than litmus to early changes in the reaction of the urine, should be employed as an indicator. The appearance of a yellow color upon its addition to the urine is the sign to suspend further administration of alkali.

Glucose is indicated in conditions with ketosis due to carbohydrate deficiency, *providing the organism can assimilate it.*

Clinical Methods.—As noted above, measurement of one variable will be inadequate exactly to determine the state of acid-base equilibrium. As long, however, as the pH is normal, which is the usual finding in most pathological conditions, including mild acidosis, one determination will suffice. Clinical methods comprise tests for whole blood or *plasma CO_2 or bicarbonate*, alveolar CO_2 tension, bicarbonate tolerance, pH of blood or urine, Sellard's test, NH_3 quotient of urine, or presence of abnormal acids (particularly acetone bodies) in blood or urine. The first two methods are the ones of choice, particularly the first, as, by it, one can estimate the

reserve of the very important blood buffer, bicarbonate, and its result closely indicates the total buffers. It is the standard, and does not require the cooperation of the patient. Women and children are especially susceptible to acidosis since their plasma CO_2 capacity is normally lower than that of men—about 8 vol. % less with women, and about 10 vol. % less with children. Normal values for men were given as 77–53 vol. %; 53–40 means mild acidosis without symptoms; 40–30, a moderate to severe acidosis possibly with symptoms; less than 30, severe with symptoms; 20 is usually fatal.

The *alveolar CO_2 tension* is a practical measure of the blood bicarbonate, although not necessarily accurate, as noted above. The cooperation of the patient is usually required, and results are influenced by many conditions, e.g., pathological processes, especially pulmonary affections and advanced cardiac disease, drugs, emotion, atmospheric variations, position of body, digestion, etc. The tension in infants is 3–5 mm. lower than in adults. For the latter, 40–45 mm. is normal, 30–35 indicative of mild acidosis, 20 mm. means imminent danger, and 8–10 mm. may be observed in coma.

The *tolerance for bicarbonate* is a very convenient and practical measure of acidosis, and means the dose of $NaHCO_3$ required to produce a urine alkaline or amphoteric to litmus. A normal finding is 5–10 grams; 20 is required with mild, 30–40 with more severe, and more than 40 Gm. with extreme degrees of acidosis. In coma, it is usually impossible to produce an alkaline urine.

Determination of the pH is clinically unsatisfactory especially in the urine, where it is particularly unreliable. In the blood, the change is small and late, and results lack uniformity by reason of the technique.

Certain *changes in the urine* are recognized and acceptable as indirect evidence of acidosis, but these changes are not synonymous with acidosis, being dependent in part upon renal integrity and other factors. The NH_3 quotient of urine (ammonia nitrogen: total nitrogen), as usually determined with patient on a mixed diet, is normally about 5%. Values of 10–40%, occur in acidosis. It may be increased by diet, disturbances of protein metabolism, ammoniacal fermentation, etc., and there may be no increase in certain diseases with acidosis. The ammonia probably does protect the blood alkali, but its efficacy is intimately associated with renal function, inasmuch as Nash and Benedict have presented strong evidence to the effect that urinary and blood NH_3 is the product of an active synthetic function of the kidneys themselves. Acetone bodies in the urine (*ketonuria*), in the blood (*ketosis*), or in the breath (sweetish odor, however, is not due to acetone), have diagnostic value but are poor indices of the severity of, and may be absent in, acidosis. They result especially from abnormal and incomplete oxidation of fats. Acetone and diacetic acid have the same significance; a progressive increase gives a grave prognosis, and it is generally considered that the presence of β-hydroxybutyric acid indicates greater severity. The qualitative test for acetone bodies in the breath is sensitive, and yields positive results earlier than Gerhardt's test on urine. It is said that the NH_3 parallels the acetone bodies and is a better measure of ketosis.

Bicarbonate.—Van Slyke determines the bicarbonate (*CO_2 combining power:* CO_2 capacity) either by a gas method for whole blood or plasma, or a titration method for plasma. We have found both methods very satisfactory and essentially follow his technique, but prefer the gas method, considering it less subject to technical errors.

Collect venous blood with the apparatus shown in Fig. 202, or by means of an all-glass syringe containing a few crystals of potassium oxalate, discharging it as soon as possible beneath paraffin oil in a centrifuge tube. Loss of CO_2 to the air is to be prevented until plasma is in apparatus. Centrifuge as soon as possible, and pipette the clear plasma into a separatory funnel, or store in the refrigerator under paraffin oil. Attach a bottle of glass beads (see Fig. 206), and after a *normal* inspiration, blow one complete expiration through the apparatus, current passing from beads to funnel. Close both ends of funnel just before finish. Distribute plasma in a thin layer by rotation, and saturate for two minutes (equilibration).

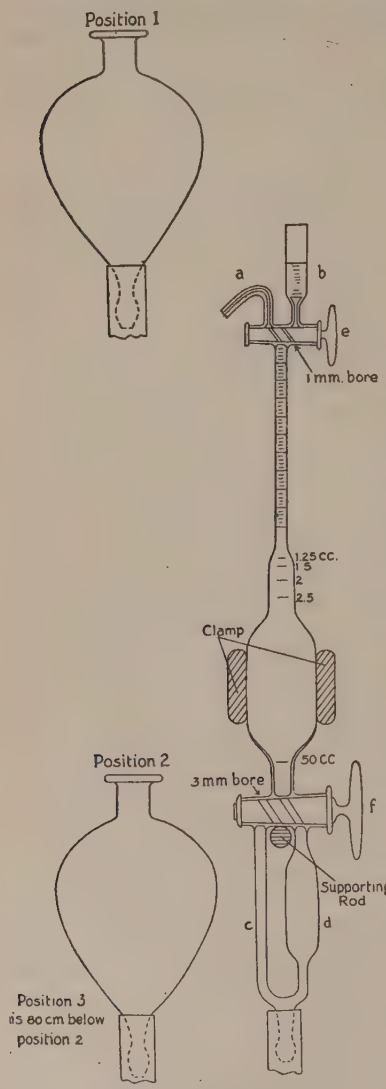

FIG. 205.—Van Slyke's plasma bicarbonate apparatus.

Fill entire apparatus (Fig. 205) with mercury, and close stopcock *e*. Put 1 cc. CO_2-free H_2O (see p. 687) in cup *b*. With leveling-bulb in "Position 2" and cock *f* as shown, slightly open cock *e*, and run 1 cc. of plasma beneath water in cup, the tip of the pipette being at the bottom of the cup and the plasma flowing practically directly into the burette. Add a drop of octyl alcohol. When capillary above cock *e* contains only the alcohol, run 0.5 cc. lactic acid (1 vol. of concentrated acid of specific gravity of about 1.2 diluted with 9 vol. H_2O) into apparatus, and stop when 2.5-cc. mark is reached by the Hg. Close cock *e*, and seal with a drop of Hg in the cup *b*. Place leveling-bulb in "Position 3," and allow Hg to run down to 50-cc. mark. Close cock *f*, remove apparatus from clamp, and mix contents by inversion 15 times. Replace in clamp, open cock *f*, and draw fluid into *d*, leaving capillary of *f* full, and allowing no gas to follow. Reverse *f*, raise leveling-bulb, and allow Hg to enter from *c* and rise slowly, and *without oscillation*. With Hg in leveling-bulb higher than that in the apparatus by one-thirteenth of the height of the water column on the mercury and above cock *f*, read the gas volume.

Calculate volumes per cent. CO_2 by use of Table, p. 716. The table corrects for temperature, pressure, and other factors.

Immediately cleanse apparatus with CO_2-free H_2O, and let stand filled with water.

Total CO_2 in plasma is determined in the same manner, but Table, p. 717, is used for correction of gas volume as read. The plasma is not equilibrated with alveolar air, but is pipetted directly into cup from beneath the paraffin oil. In the calculation subtract the proper amount in third column from the observed gas volume, and multiply by the factor in the fourth column.

For whole blood, the technique is somewhat modified: The whole blood is equilibrated, and 1 cc. is placed in cup as for plasma; stir water in cup when only about 0.5 cc. remains; read gas volume, and then introduce a little N/2 NaOH via the cup under *very slight* negative pressure; this dissolves the CO_2, the amount of which is

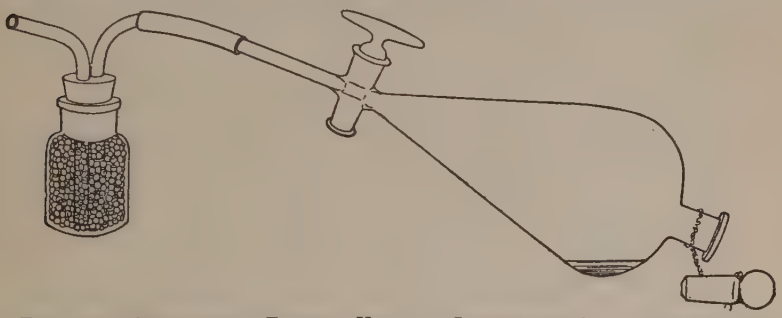

FIG. 206.—SEPARATORY FUNNEL USED IN SATURATING BLOOD PLASMA WITH CARBON DIOXIDE. The bottle contains glass beads. (*Journal Biological Chemistry*, 30, 289, 1917.)

indicated by the loss in gas volume; that being corrected in same manner as for total CO_2 in plasma, but omitting the subtraction of third column. The result is CO_2 capacity. For total CO_2, the blood is not equilibrated.

Some use arterial blood, and Stadie has shown that arterial puncture is a safe clinical procedure. The technique is slightly modified from that for venous puncture. Use a needle 1–2 mm. in diameter, very sharp, and with a 45° bevel. Anaesthetize locally with procaine, insert needle through skin at an angle of 45°, and plunge into radial artery at wrist at point of maximum pulsation. Follow withdrawal of needle by obliterating pressure for one to two minutes, and then moderate pressure with a gauze compress for two to three hours.

Alveolar CO_2 tension.—This is readily measured in the alveolar air by the usual methods of gas analysis. Marriott's method, while subject to many sources of error, is useful, and, by the use of a special outfit, can be utilized practically at the bedside. This outfit being simple, inexpensive, and accompanied by directions for use, its purchase is probably preferable for clinical purposes to the use of more elaborate apparatus in the laboratory. The sample obtained at the end of a full expiration following a *normal* inspiration should approximate the CO_2 tension in arterial blood; one obtained by rebreathing is closer to that of venous blood.

Reaction of blood.—Marriott has also improved the Levy, Rowntree, and Marriott method for clinical determination of the pH of the blood. Cullen claims an accuracy

Table for Calculation of Carbon Dioxide Combining Power of Plasma[1]

Observed vol. gas × Barometer / 760	Cc. of CO_2 reduced to 0°C 760 mm. bound as bicarbonate by 100 cc. of plasma				Observed vol. gas × Barometer / 760	Cc. of CO_2 reduced to 0°C 760 mm. bound as bicarbonate by 100 cc. of plasma			
	15°	20°	25°	30°		15°	20°	25°	30°
0.20	9.1	9.9	10.7	11.8	0.60	47.7	48.1	48.5	48.6
1	10.1	10.9	11.7	12.6	1	48.7	49.0	49.4	49.5
2	11.0	11.8	12.6	13.5	2	49.7	50.0	50.4	50.4
3	12.0	12.8	13.6	14.3	3	50.7	51.0	51.3	51.4
4	13.0	13.7	14.5	15.2	4	51.6	51.9	52.2	52.3
5	13.9	14.7	15.5	16.1	5	52.6	52.8	53.2	53.2
6	14.9	15.7	16.4	17.0	6	53.6	53.8	54.1	54.1
7	15.9	16.6	17.4	18.0	7	54.5	54.8	55.1	55.1
8	16.8	17.6	18.3	18.9	8	55.5	55.7	56.0	56.0
9	17.8	18.5	19.2	19.8	9	56.5	56.7	57.0	56.9
0.30	18.8	19.5	20.2	20.8	0.70	57.4	57.6	57.9	57.9
1	19.7	20.4	21.1	21.7	1	58.4	58.6	58.9	58.8
2	20.7	21.4	22.1	22.6	2	59.4	59.5	59.8	59.7
3	21.7	22.3	23.0	23.5	3	60.3	60.5	60.7	60.6
4	22.6	23.3	24.0	24.5	4	61.3	61.4	61.7	61.6
5	23.6	24.2	24.9	25.4	5	62.3	62.4	62.6	62.5
6	24.6	25.2	25.8	26.3	6	63.2	63.3	63.6	63.4
7	25.5	26.2	26.8	27.3	7	64.2	64.3	64.5	64.3
8	26.5	27.1	27.7	28.2	8	65.2	65.3	65.5	65.3
9	27.5	28.1	28.7	29.1	9	66.1	66.2	66.4	66.2
0.40	28.4	29.0	29.6	30.0	0.80	67.1	67.2	67.3	67.1
1	29.4	30.0	30.5	31.0	1	68.1	68.1	68.3	68.0
2	30.3	30.9	31.5	31.9	2	69.0	69.1	69.2	69.0
3	31.3	31.9	32.4	32.8	3	70.0	70.0	70.2	69.9
4	32.3	32.8	33.4	33.8	4	71.0	71.0	71.1	70.8
5	33.2	33.8	34.3	34.7	5	71.9	72.0	72.1	71.8
6	34.2	34.7	35.3	35.6	6	72.9	72.9	73.0	72.7
7	35.2	35.7	36.2	36.5	7	73.9	73.9	74.0	73.6
8	36.1	36.6	37.2	37.4	8	74.8	74.8	74.9	74.5
9	37.1	37.6	38.1	38.4	9	75.8	75.8	75.8	75.4
0.50	38.1	38.5	39.0	39.3	0.90	76.8	76.7	76.8	76.4
1	39.1	39.5	40.0	40.3	1	77.8	77.7	77.7	77.3
2	40.0	40.4	40.9	41.2	2	78.7	78.8	78.7	78.2
3	41.0	41.4	41.9	42.1	3	79.7	79.6	79.6	79.2
4	42.0	42.4	42.8	43.0	4	80.7	80.5	80.6	80.1
5	42.9	43.3	43.8	43.9	5	81.6	81.5	81.5	81.0
6	43.9	44.3	44.7	44.9	6	82.6	82.5	82.4	82.0
7	44.9	45.3	45.7	45.8	7	83.6	83.4	83.4	82.9
8	45.8	46.2	46.6	46.7	8	84.5	84.4	84.3	83.8
9	46.8	47.1	47.5	47.6	9	85.5	85.3	85.2	84.8
0.60	47.7	48.1	48.5	48.6	1.00	86.5	86.2	86.2	85.7

[1] The temperature figures at the heads of columns represent the room temperature at which the samples of plasma are saturated with alveolar carbon dioxide and analyzed. It is assumed that both operations are performed at the same temperature. The figures have been so calculated that, regardless of the room temperature at which saturation and analysis are performed, the table gives the volume (reduced to 0°C, 760 mm.) of carbon dioxide that 100 cc. of plasma are capable of binding when saturated at 20° with carbon dioxide at approximately 41 mm. tension. If the figures in the table are multiplied by 0.94 they give, within 1 or 2 per cent., the carbon dioxide bound at 37°C.

TABLE FOR OTHER BLOOD GAS DETERMINATIONS

Room temperature, °C.	For oxygen determinations	For total CO_2 determinations	
	Correction for reduction to 0°C., 760 mm.	Correction for dissolved air	Correction for reduction to 0°C., 760 mm.
15	$46.6 \times \frac{\text{Barometer}}{760}$	0.052	$100.2 \times \frac{\text{Barometer}}{760}$
16	$46.4 \times \frac{\text{Barometer}}{760}$	0.051	$99.5 \times \frac{\text{Barometer}}{760}$
17	$46.2 \times \frac{\text{Barometer}}{760}$	0.050	$98.9 \times \frac{\text{Barometer}}{760}$
18	$45.95 \times \frac{\text{Barometer}}{760}$	0.049	$98.3 \times \frac{\text{Barometer}}{760}$
19	$45.75 \times \frac{\text{Barometer}}{760}$	0.048	$97.8 \times \frac{\text{Barometer}}{760}$
20	$45.5 \times \frac{\text{Barometer}}{760}$	0.047	$97.2 \times \frac{\text{Barometer}}{760}$
21	$45.3 \times \frac{\text{Barometer}}{760}$	0.046	$96.6 \times \frac{\text{Barometer}}{760}$
22	$45.05 \times \frac{\text{Barometer}}{760}$	0.045	$96.0 \times \frac{\text{Barometer}}{760}$
23	$44.85 \times \frac{\text{Barometer}}{760}$	0.045	$95.4 \times \frac{\text{Barometer}}{760}$
24	$44.6 \times \frac{\text{Barometer}}{760}$	0.044	$94.8 \times \frac{\text{Barometer}}{760}$
25	$44.4 \times \frac{\text{Barometer}}{760}$	0.043	$94.2 \times \frac{\text{Barometer}}{760}$
26	$44.15 \times \frac{\text{Barometer}}{760}$	0.042	$93.6 \times \frac{\text{Barometer}}{760}$
27	$43.9 \times \frac{\text{Barometer}}{760}$	0.041	$93.1 \times \frac{\text{Barometer}}{760}$
28	$43.65 \times \frac{\text{Barometer}}{760}$	0.040	$92.4 \times \frac{\text{Barometer}}{760}$
29	$43.4 \times \frac{\text{Barometer}}{760}$	0.040	$91.8 \times \frac{\text{Barometer}}{760}$
30	$43.15 \times \frac{\text{Barometer}}{760}$	0.039	$91.2 \times \frac{\text{Barometer}}{760}$

of ±0.02 pH for his method on plasma or serum. However error in handling the method in the average laboratory may vitiate the results.

Acetone bodies.—Briggs and Shaffer base a qualitative test for acidosis upon the fact that the acetone bodies in the breath are proportional to their concentration in the blood. Place some ice-cold, distilled H_2O in a large test tube, and exhale through the same for one to two minutes, using a glass tube. Then add 10 to 20 cc. Scott-Wilson reagent, and let stand. Normally, the contents of the tube remain clear; a positive result is a faint to deep opalescence, or even a precipitate, after a few minutes. The test is roughly quantitative.

The reagent is prepared by dissolving 1 Gm. mercuric cyanide in 60 cc. H_2O in a heavy-walled glass jar, and adding a solution of 18 Gm. NaOH in 60 cc. H_2O; add slowly, while stirring constantly and vigorously with a heavy glass rod, 0.29 Gm. $AgNO_3$ dissolved in 40 cc. H_2O; the mixture should be clear and may then be used at once, but, if turbid, it must stand and settle for three to four days, the clear, supernatant fluid then being siphoned off. The solution gradually deteriorates, but remains serviceable for qualitative tests for several months.

Sellard's test for serum acidosis is quite reliable and simple. Add 1 cc. serum drop by drop to 25 cc. of *neutral* absolute alcohol to precipitate the protein, which interferes with a sharp end-reaction. Filter, add 3 to 4 drops neutralized phenolphthalein solution to filtrate, and evaporate in porcelain dish on water bath. Every piece of apparatus must be perfectly dry, and the steam of the bath quite low, to avoid absorption of H_2O by the alcohol. Normally, the dark pinkish tinge of the sediment remains at least one hour; with acidosis, it disappears in a few minutes.

Diastase.—Winsløw's technique is satisfactory, using serum or whole blood instead of urine (see p. 725). Normal figures are 8–64 units. (See p. 614 for discussion.)

Occult Blood

When the presence of blood in the faeces, gastric contents, urine or body fluids, is suspected but cannot be recognized by macroscopic or microscopic methods, it is necessary to resort to spectroscopic or chemical tests. These tests are, however, individually unsatisfactory. The spectroscopic method is not delicate, the haemin-crystal method does not give uniform results, and the various color tests, although very sensitive, are given by many substances other than blood. Consequently, it may be said that, with the color tests, it is negative results that are significant, and with other than the color tests it is positive findings that are informative. Serological tests are the most satisfactory medicolegally.

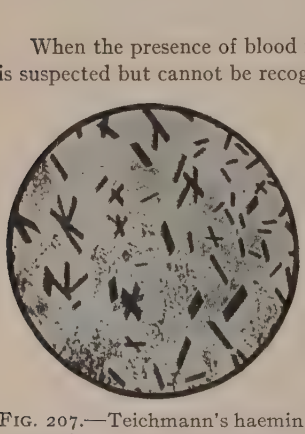

Fig. 207.—Teichmann's haemin crystals. (*Todd after Jakob.*)

Haemin Crystal Test (Teichmann).—Prepare a solution (stable) of 0.1 Gm. each of KI, KBr, and KCl in 100 cc. glacial acetic acid. Mix a few drops with some of the material on a slide, apply a cover glass, and *gently* warm until bubbles begin to appear. Then cool *slowly*, and examine for the characteristic dark-brown crystals.

Haemochromogen Crystals (Donogány).—Mix one drop each of suspected fluid, pyridin, and 20% NaOH on slide, and let dry. If positive, radiating needles will form after several hours.

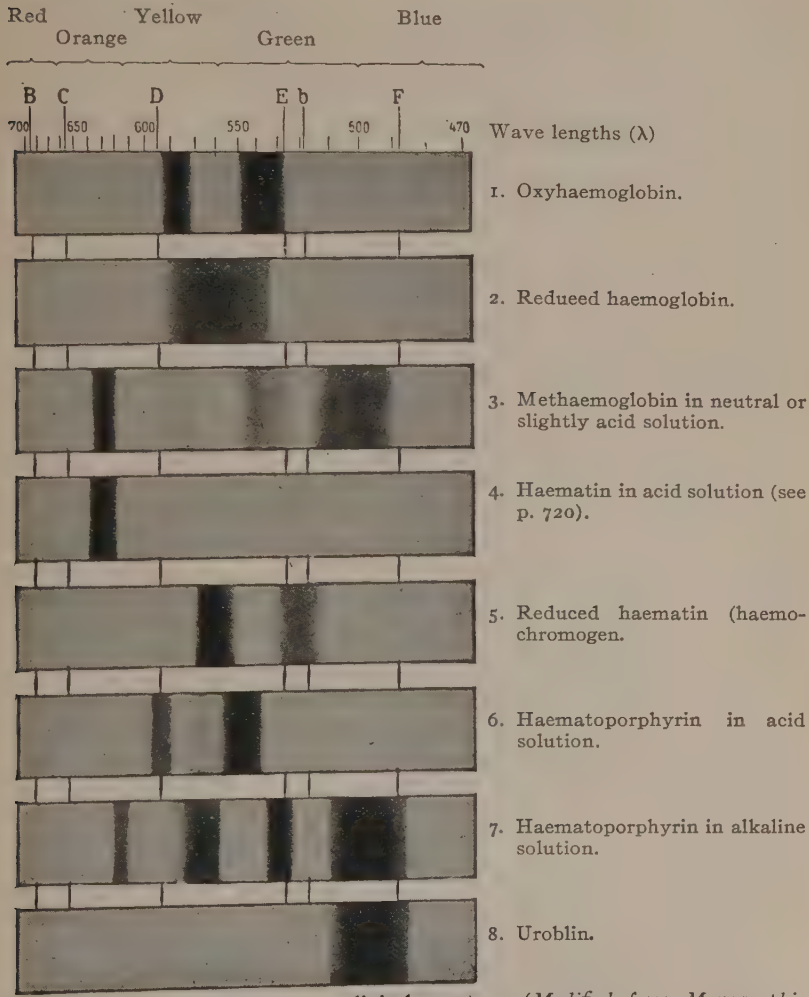

FIG. 208.—The most important clinical spectra. (*Modified from Monographic Medicine.* D. Appleton & Co., New York.)

Spectroscopic Tests.—These depend upon the recognition of the characteristic absorption spectra of haemoglobin or its derivatives (Fig. 208). The degree of concentration influences their appearance, and one should start with a relatively concentrated solution, diluting cautiously until the bands are typical.

The small, direct-vision (hand) spectroscope suffices. A wave-length scale is a convenient attachment. Daylight or strong artificial light (such as the "daylite" lamp) is used. Have solution in a small test tube or, preferably, a flat cell with a thickness of about 1 cm. Before use, focus Frauenhofer's lines sharply.

Reducing agents, such as ammonium sulphide or Stokes' solution, are employed. These must be fresh, and, before use, the sulphide must be warmed to about 50°C. To prepare Stokes' solution, dissolve 3 Gm. $FeSO_4$ in cold H_2O, add cold aqueous solution of 2 Gm. tartaric acid, and make up to 100 cc. Immediately before use, add strong, NH_4OH until precipitate first formed is dissolved.

Material that is uncontaminated, relatively fresh and in relatively concentrated aqueous solution may give any or all of the upper three spectra, a few drops of reducer changing the first to the second.

If the material is older, dissolve the suspected stain in 1–2 cc. of 10% NaOH, heat almost to boiling, cool, and add a few drops of reducer. Examination shows Spectrum 5.

Old and relatively uncontaminated stains are dissolved in KCN solution and warmed to 40°C. Examination shows cyanhaematin (poorly defined bands resembling Spectrum 2). Reduction with ammonium sulphide gives cyanhaemochromogen (similar to Spectrum 5). In cyanide poisoning, blood pigment in walls or lumen of stomach shows cyanhaematin.

With very old and relatively uncontaminated material, if insoluble in KCN solution, a small fragment is mixed with a few drops concentrated H_2SO_4, and crushed and rubbed between two glass slides. Examination shows Spectrum 6.

If very old and much mixed with other material, pulverize, mix with a few cubic centimeters concentrated H_2SO_4, and let stand twenty-four hours. Filter through glass wool or asbestos, and pour filtrate into 10 times its volume of distilled water. Wash the brown precipitate several times, and dry. Dissolve in a mixture of equal parts of absolute alcohol and strong NH_4OH, and filter. Examination shows Spectrum 7, which, upon acidification, will change to a faint Spectrum 6.

It is better, however, especially with much contamination, to prepare an ethereal, acid extract. After having ground the material thoroughly with water, if it is not already in liquid form, mix gently with an equal volume of neutral ether. Reject ether extract, and, to 10 cc. of residue, add 3 to 5 cc. of glacial acetic acid. Shake thoroughly with an equal volume of ether. If the ether does not separate readily, mix gently with a few drops of alcohol. Remove ethereal extract, and evaporate it to a small bulk for use in tests. Examination will show spectrum of acid haematin, which, however, in ethereal solution, resembles Spectrum 3 more than 4. Make alkaline with strong NH_4OH, cool, mix well, let separate, and add a few drops of reducer to the ammoniacal extract. Examination shows Spectrum 5.

The spectra of carbon monoxide haemoglobin and of nitric oxide (N_2O_2) haemoglobin are similar to that of oxyhaemoglobin, but reducing agents will not give Spectrum 2, provided the haemoglobin is more than 20% saturated with CO, the usual condition in serious poisoning. Chemical tests for the presence of CO are probably more satisfactory than spectroscopic. To 2 drops blood, add 40 drops water, then 40 drops 30% NaOH, and mix. With CO-haemoglobin, bright-red precipitate; with oxyhaemoglobin, dirty brown. Controls of normal blood and of same saturated with illuminating gas are useful.

Donogány's method increases the delicacy of the spectroscopic test, and is also a color test. Dissolve the pigment with 20% NaOH, add fresh pyridin and, if necessary, fresh ammonium sulphide. Filter. The filtrate will be more or less orange-red according to blood content, and will show Spectrum 5. (Fig. 208.)

Color Tests.—The reliability of these may be enhanced by the use of methods which involve the removal or destruction of interfering substances. In such a method, the original aqueous solution is boiled for 15 to 20 seconds, and the acid ethereal extract is prepared as previously described. This extract is dropped on filter paper, the reagents being applied to the moistened spot. The delicacy of these several tests is variable, being in aqueous solution approximately 1–25,000 for the guaiac and aloin, and 1–250,000 for the benzidine test. In material of animal origin, the delicacy may be much less, especially in faeces.

(*a*) Treat moist spot with a few drops of freshly prepared 2% alcoholic solution of *guaiac* resin, and then a few drops of hydrogen peroxide. A blue color is "positive."

(*b*) Treat moist spot with a few drops of 3% *aloin* in 70% alcohol, and then with ozonized turpentine (turpentine that has stood for a few days in an open vessel in sunlight). A purplish-red color within 10 minutes is "positive."

(*c*) Drop on moist spot 2 drops glacial acetic acid, a few crystals of *benzidine* (preferably white), and finally 2 drops hydrogen peroxide. Greenish-blue color if positive.

G—CHEMICAL EXAMINATION OF SPINAL FLUID

Spinal fluid probably should be regarded as a dialysate rather than a secretion. In harmony with this concept the nonprotein nitrogen constituents and sugar will be found to vary with those of the blood but with considerable lag in time and degree of fluctuation due to the fact that spinal fluid is formed slowly (5–30 cc. per hour) and mixed with that previously and subsequently formed. Spinal fluid sugar and chlorides appear to be the two constituents which are of diagnostic importance in acute and chronic inflammatory conditions of the brain and spinal cord. Luetic involvement is not marked by definite deviation from the normal. Spinal fluid sugar should be determined on fasting (overnight) patients and compared with blood sugar. It varies normally between 50 and 80 mg. per 100 cc. spinal fluid. In the absence of hypoglycaemia spinal fluid sugars below 50 mg. per 100 cc. nearly always indicate an infection of the meninges. In acute purulent meningitis spinal fluid sugar drops in 24 hours to 20 or below. In localized meningitis (secondary to mastoiditis) the drop may be somewhat slower. In acute tuberculous meningitis sugar drops slowly perhaps reaching 35 mg. in the first week, 20 mg. the second, and 15 mg. the third week.

Sugars above 80 occur in epidemic encephalitis and a variety of other conditions. Such spinal fluid sugars are usually associated with hyperglycaemia which is not uncommon in cerebral conditions.

Chlorides normally vary from 720 to 750 mg. per 100 cc. estimated as sodium chloride. Values lower than 600 mg. seldom occur except in tuberculous meningitis. Levels of 630 to 680 are commonly found in acute purulent meningitis. Chlorides are normal in epidemic encephalitis and brain tumor (uncomplicated by septicaemia or meningitis). Chlorides are slightly diminished in acute anterior poliomyelitis (670–710). According to Smith and Dailey, low (below 50) or progressively falling

spinal fluid sugar plus low chlorides (under 620) and a moderate increase in cells (400–500 lymphocytes) is characteristic of tuberculous meningitis.

Methods for the determination of the nonprotein nitrogen constituents, sugar and chlorides in spinal fluid are identical with those used for the Folin-Wu tungstic acid filtrate, the spinal fluid being diluted 1 in 10 with distilled water.

Method for the quantitative determination of protein in cerebrospinal fluid (Denis and Ayer).—Into a test tube of about 4 cc. capacity, 0.6 cc. of spinal fluid is measured. To this is added 0.4 cc. of distilled water and 1 cc. of a 5 per cent solution of sulphosalicylic acid. The contents of the tube is then mixed by inversion (but not by violent shaking) and after being allowed to stand for five minutes the suspension is read by means of a suitable colorimeter against a standard protein suspension prepared at the same time as the unknown. Before reading the standard against the unknown, the standard solution should be placed in both cups and several readings made. In fluids of extremely high protein content, such as may be encountered in cases of spinal cord compression, meningitis, etc., it is sometimes necessary to make a preliminary dilution with water as even 0.1 cc. of such fluids may contain too much protein to read against the standard.

Preparation of standard for total protein determination (Ayer and Foster).—Twenty cc. of fresh normal human blood serum are diluted to 200 cc. with 15% solution of sodium chloride in a volumetric flask and filtered. This filtrate is the concentrated standard.

The total nitrogen of this filtrate is determined by macro-Kjeldahl on 40 cc. The nonprotein nitrogen is determined on the original undiluted serum by the micro-Kjeldahl method of Folin and this figure divided by ten is subtracted from the total nitrogen to obtain protein nitrogen. Protein nitrogen multiplied by 6.25 gives the protein content of the concentrated standard.

This concentrated standard is diluted with distilled water to make the dilute standard containing 30 mgs. protein per 100 cc.

These standards are preserved with a few crystals of thymol and kept on ice except when in use. In this way we have kept the concentrated standards for more than six and the dilute standards more than twelve months without appreciable change in protein content.

H—CHEMICAL EXAMINATION OF URINE

A twenty-four-hour specimen of urine is necessary for the results of a chemical examination to be significant. The amount secreted normally varies with fluid intake and other factors. Women pass about 200 cc. less than men per day; children, in proportion to body weight, excrete about 4 times as much as adults. It is well to bear in mind that the color is markedly affected by certain drugs and foods.

To prevent decomposition of a specimen, a matter of importance especially in examinations for casts, albumin and other nitrogenous constituents, the urine is preferably kept on ice. The next best method is to add toluene to about 2%. Xylol, boric acid (0.1%), camphor and chloral are less satisfactory as preservatives. Formalin, chloroform, thymol, salicylic acid and $HgCl_2$ will interfere with certain tests.

Reaction.—The twenty-four-hour sample is normally acid to litmus, but at certain times, especially after meals, isolated specimens may be alkaline. This test

with litmus suffices clinically, and quantitative estimations of acidity are not of real diagnostic value. Folin gives, for twenty-four hours, an average normal titration value of 617 cc. N/10 acid, using the following technique: In each of 2 small flasks, place 25 cc. undiluted and carefully preserved urine; add 15 Gm. neutral potassium oxalate, 1 to 2 drops 1% phenolphthalein, and shake thoroughly for one to two minutes. Titrate immediately with N/10 NaOH (end-point is the first permanent color change). Second flask is used as control.

Hydrogen-ion concentration.—Acidity of urine as determined by titration does not measure the true reaction, which depends upon the ionic hydrogen present, although the results obtained may often correspond to some degree with the pH. As noted elsewhere, a rough estimate of the pH of the unknown (urine in this case) can be obtained by systematically testing it with different indicators, since reference to the list (p. 689) will show the pH at which their full acid or alkaline color may be expected. The hydrogen-ion concentration may be more accurately determined by using the Barnett-Chapman technique (p. 692) or by comparison with the proper buffer mixtures (p. 690). Indicators, methyl red (4.4–6.0), bromcresol purple (5.2–6.8), or phenol red (6.8–8.4) usually suffice. The urine should be collected and kept under oil. Precautions with reference to backing (noted on p. 693) must be observed. The urine should be diluted 1 to 5, CO_2 free distilled water being used throughout. The normal pH values (as given by Hawk) lie between 5.5 and 8.0 with a mean value of 6.0. For a strictly vegetarian diet the average is 6.64. In cardiorenal disorders the pH may be 5.3. In most pathological conditions the hydrogen-ion concentration is increased (pH decreased) (Hawk).

Specific Gravity.—A very low specific gravity is usually due to excessive fluid intake, and should be viewed with suspicion in case of a candidate appearing for physical examination, since pathological constituents often escape detection in the resulting dilution.

Total Solids.—Long determines these in grams per liter by multiplying the two final figures of the specific gravity by 2.6 (use 1.6 for small children).

Total Nitrogen. (Folin-Denis).—Use NH_3-free H_2O (see p. 702) throughout. Remove albumin (see p. 727) if present, and dilute urine so that 1 cc. contains 0.7–1.5 mg. N, a sufficiently accurate dilution being 1–5 for specific gravity of less than 1.018, 1–10 for 1.018–1.030, and 1–20 for higher.

In a Pyrex test tube (190 × 25 mm.), place 1 cc. diluted urine, 1 cc. *undiluted* acid-digestion mixture (p. 701), and a quartz pebble, or a piece of platinum. Heat over micro-burner with the top of the burner less than 1 cm. below tube, until *dense*, white fumes appear (2–5 minutes). Then cover with watch glass, regulate flame so that but little of fumes escape, and heat until the color becomes clear blue or green (0.5–3 minutes). Continue heat 0.5–1 minute. Remove flame, and cool in place two minutes. Add H_2O, and rinse quantitatively into a 200-cc. volumetric flask with about 150 cc. H_2O. Prepare standard by placing in a 200-cc. volumetric flask 1 cc. of same acid-digestion mixture, 20 cc. working N standard (p. 701), and about 125 cc. H_2O.

To each flask, add 30 cc. Nessler's solution (see p. 701), fill to marks with H_2O, mix, clear by centrifuge if necessary, and compare in colorimeter.

Calculation (if diluted 1–10): $\frac{S}{U} \times 10 =$ mg. N per cc. urine.

Urea.—The following Folin-Youngburg technique is simple and accurate. Use NH_3-free H_2O (see p. 702) throughout. Dilute urine 1–10 (1–5 if specific gravity low). Remove NH_3 by agitating 25 cc. with 4 Gm. dry permutit for five minutes; let settle 15 to 30 seconds; decant, centrifuging if not clear. Place 1 cc. of this diluted urine in test tube, add 1 cc. urease solution (p. 702) and 1 drop pyrophosphate mixture (p. 702), and digest in water at about 50°C. for five minutes. Transfer quantitatively to a 200-cc. volumetric flask. Prepare standard by placing 20 cc. working N standard (p. 701) and 1 cc. urease solution in another 200-cc. flask. To each flask, add H_2O to about 150 cc., and then 20 cc. Nessler's solution (p. 701). Dilute to marks, mix, and compare in colorimeter.

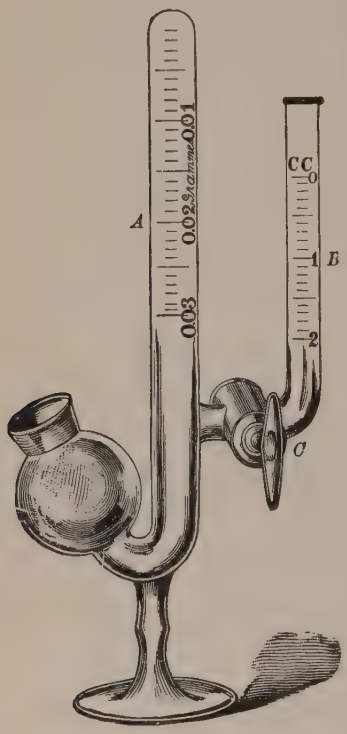

Fig. 209.—Doremus-Hinds ureometer.

Calculation (if diluted 1–10): $\frac{S}{U} \times 21.4 =$ mg. urea per cc. urine.

Permutit can be used repeatedly if following precautions are taken. After use, rinse briefly and pour into container. When sufficient is collected, digest with 1% NaOH at 60°C. for about an hour to remove NH_3. Rinse with distilled H_2O and wash with 2% acetic acid until no longer alkaline to phenolphthalein. Wash further with H_2O, and dry. It is well to discard any portion that does not settle quickly and cleanly in water (like sand). Its fineness should be 60–80 mesh.

The familiar *hypobromite method* is quite inaccurate, and the results, when divided by 2.14 (the urea factor), usually represent total N more nearly than the N of the urea. It is, however, simple and rapid, and affords a rough estimate. It is convenient to use the Doremus-Hinds ureometer (Fig. 209), the scale of which usually indicates grams of urea in the volume of urine introduced. Fill the closed tube with reagent (made by dissolving 1 cc. Br in 40 cc. cold 20% NaOH; it deteriorates in a few days, even when stored in cool, dark place); slowly introduce the urine, diluted usually 2 to 4 times so that the urea will be of about 1% strength, by means of the side tube with cock; read when froth has disappeared. Albumin causes froth to be more persistent.

Ammonia (Folin-Bell).—Use NH_3-free H_2O (see p. 702) throughout. Put about 2 Gm. permutit in a 200-cc. volumetric flask, and wash down with less than 5 cc. H_2O; add 2 cc. urine, and wash down with 1–5 cc. H_2O. Shake gently but continuously for five minutes. Rinse powder to bottom of flask with 25–40 cc. H_2O, let settle, and decant supernatant fluid. Wash powder with H_2O and decant 3 suc-

cessive times, rejecting washings. Add a little H_2O to powder in flask. Prepare standard by placing 10 cc. working N standard (p. 701) in a 200-cc. volumetric flask. To each flask, add about 5 cc. 10% NaOH, and mix well; dilute quickly to about 150 cc. with H_2O, and mix well; add 10 cc. Nessler's solution (p. 701), fill to marks, mix, let stand ten minutes, and compare in colorimeter. This permutit should not be reclaimed, as it has been contaminated by Nessler's solution.

$$\text{Calculation: } \frac{S}{U} \times 0.3 = \text{mg. } NH_3 \text{ per cc. urine.}$$

Uric Acid.—Place 1 cc. urine in a centrifuge tube, add about 4 cc. H_2O and 5 cc. silver lactate solution (p. 704). Stir with a *very fine* glass rod; rinse rod with a few drops H_2O. Centrifuge two to three minutes. Test with 1 drop lactate solution, and, if it causes more precipitate, add 2 cc. lactate solution and again centrifuge. Discard supernatant fluid as completely as possible by decantation, add 4 cc. 5% NaCN (DEADLY POISON), stir until solution is perfectly clear, and transfer quantitatively to a 100-cc. volumetric flask, using 15–25 cc. H_2O. Add 5 cc. 10% sodium sulphite.

Prepare standard by placing 5 cc. uric acid standard and 4 cc. 5% NaCN in another 100-cc. flask. To each flask, add H_2O to about 40 cc., 20 cc. 20% Na_2CO_3, and mix; then add 2 cc. uric acid reagent *with shaking*, and let stand three to five minutes. Fill to marks, mix, and compare in colorimeter.

$$\text{Calculation: } \frac{S}{U} \times 0.5 = \text{mg. uric acid per cc. urine.}$$

Uric acid standard is prepared by dissolving 1 Gm. uric acid in 125 to 150 cc. 0.4% lithium carbonate, and diluted with H_2O to 500 cc. Transfer 50 cc. (100 mg. uric acid) to a liter volumetric flask, add 200 to 300 cc. water, then 500 cc. 20% Na_2SO_3 that has stood overnight and been filtered, make up to volume, and mix. Place this standard, of which 10 cc. contains 1 mg. uric acid, in 200-cc. bottles, and stopper tightly with rubber stoppers. Opened daily, it will keep at least three to four months; unopened, indefinitely.

For *uric acid reagent*, dissolve 100 Gm. sodium tungstate and 80 cc. 85% phosphoric acid in 700 cc. H_2O. Boil two or more hours, replacing lost H_2O from time to time. Allow to cool, dilute to 1 liter, and bottle.

Creatinine excretion is practically constant in amount for twenty-four-hour periods with the same individual under the same conditions. It affords, therefore, an easy method for determining whether or not the entire twenty-four-hour specimen is being submitted. Folin determines it by placing 1 cc. urine in one 100-cc. volumetric flask, and 1 cc. *stock* standard creatinine solution (p. 703) in another; to each, add 20 cc. saturated solution of *pure* picric acid and 1.5 cc. 10% NaOH, and let stand ten minutes; dilute to marks with H_2O, mix, and compare in colorimeter.

$$\text{Calculation: } \frac{S}{U} = \text{mg. creatinine per cc. urine.}$$

Diastase.—We have found Winsløw's method satisfactory. In each of a series of 10 small test tubes, place 1 cc. 1% NaCl. Add 1 cc. urine to the second tube, mix and transfer 1 cc. of mixture to third tube. Repeat down the series, and discard

the extra cubic centimeter in last tube. The first tube is the control. To each tube, add 2 cc. 0.1% soluble starch, mix, and place in water bath at 37°C. for thirty minutes. Then add 2–3 drops N/20 iodine to each tube, and mix. Determine the tube that both shows no blue and contains the least urine. Report as the number of cubic centimeters of starch solution that 1 cc. urine would digest, i.e., if the second tube is selected, it contains 0.25 cc. urine, and the report would be 8. A normal finding is 8–32. See p. 614 for discussion.

Chlorides.—Remove albumin if present (see p. 727). For an approximate determination, dilute 5 cc. urine with 50–75 cc. distilled H_2O, add 10–15 drops 10% potassium chromate, and titrate with N/10 $AgNO_3$ to a very slight pink. Calculation: 1.17 × cc. $AgNO_3$ required = mg. NaCl per cc. urine.

The Volhard-Arnold method gives accurate results, and Whitehorn applies it as for his determination of blood chlorides (p. 704). One cubic centimeter urine (to which 25 cc. distilled water is added) replaces the blood filtrate, and the cyanate and $AgNO_3$ solutions are 5 times as strong (1 cc. = 8.25 mg. NaCl). Calculation: 8.25 (5 minus cc. KCNS solution required) = mg. chlorides (as NaCl) per cc. urine.

Proteins.—*Serum albumin* and *serum globulin* usually occur together, and, in qualitative tests, have practically the same clinical significance, both being shown by the tests below. The latter constitutes 10–75% of total proteins; the quantity of the albumin divided by the quantity of globulin gives the *albumin quotient.*

Simon probably expresses the general opinion when he says, "It may be safely asserted that a transitory, intermittent, and cyclic albuminuria is not infrequently observed in apparently healthy individuals, but that the facts so far brought forward do not warrant the assumption that such forms of albuminuria are physiological. The occurrence of such albuminuria unquestionably demonstrates a certain insufficiency of the renal epithelium."

The presence of cylindruria and albuminuria does not affirm organic disease of the kidney, nor does their absence deny it; nor does albuminuria necessarily parallel the severity of the lesion; but if there is also an increased uric acid in the blood, the syndrome would rather definitely suggest an organic lesion. As the "physiological" albuminurias and cylindrurias are essentially transitory in nature, repeated examinations are indicated when albumin or casts is found. Persistently positive findings speak for renal disease, although mortality statistics would indicate that faintest possible traces of albumin without cylindruria are of no significance between the ages of fifteen and twenty-four years.

The gravity of a finding of albumin varies directly with its amount, especially if associated with casts. Definite conclusions regarding type of lesion require a consideration of the urine as a whole, and the interpretation of findings should be made by the clinician in charge of the patient.

The amount of albumin varies during the day, and afternoon specimens are preferable, especially those following exercise or meals. For the tests, the urine must be perfectly transparent, which condition may usually be attained by filtration. As a last resort, mixing with a liberal amount of U. S. P. purified talc and filtering may be done, but this will remove traces of albumin. When the urine to be examined is concentrated, it is well to dilute it prior to making the tests. Normal urine probably contains about 0.005% serum albumin, which amount will not afford positive results with the tests given. Five to ten grams of albumin per twenty-four hours is con-

sidered a moderate amount. One hundred thousand leukocytes per cubic millimeter will be responsible for about 0.1%.

Heat and acetic acid test.—Boil the upper portion of half a test-tubeful of urine and add 3–10 drops 5% acetic acid, boiling between each drop. If the urine is alkaline or poor in salts, first add one-fifth volume saturated solution of NaCl. A turbidity in the boiled portion that clears with the acid is ordinarily due to carbonates (effervesces) or phosphates. If it persists, it signifies albumin; and the more brown the color the more acute the condition. Purdy adds NaCl as above and 5–10 drops 50% acetic acid before boiling, his modification being more reliable, as it is said to rule out "nucleoprotein" and is also more delicate.

Heller's test is less delicate, and consists in carefully superimposing a layer of urine upon colorless concentrated HNO_3, and letting stand more than three minutes. The U-tube (Fig. 12) may be employed, but interpretation is more satisfactory with a larger vessel, such as a conical glass. Albumin gives a white layer at surface of contact. Other "rings," however, may appear. At surface of contact, a crystalline "ring" of urea nitrate may develop, or one due to resins, the latter being soluble in ether; a white one 5–10 mm. higher is due to "nucleoprotein" or urates; below surface of contact, a reddish "ring" is due to normal coloring matter, a mahogany color to urobilin, while a play of color (see p. 731) is due to bile pigment; between this colored layer and that of albumin, indican produces a violet color. Dilution of urine to a specific gravity of 1.005 tends to eliminate these nonalbuminous "rings."

To *remove albumin*, bring a definite volume of urine just to the boiling point, add a few drops of diluted acetic acid, avoiding an excess, boil two or three minutes, and cool. Filter, restore to original volume with water run through the filter, and check efficacy by testing filtrate for albumin. If urine is alkaline or poor in salts, first add NaCl as above, and, in former case, acidify faintly with acetic acid.

Quantitative tests for albumin that are clinically convenient are only approximately accurate, but those to be given are sufficiently so for clinical purposes. *Purdy's* is generally the most satisfactory. He places 10 cc. urine in a graduated centrifuge tube, adds first 2 cc. 50% acetic acid, and, second, 3 cc. 10% potassium ferrocyanide, mixes gently by inversion, and lets stand ten minutes. Centrifuge for exactly three minutes at 1500 revolutions per minute in a centrifuge having a radius of 6¾ inches with tubes extended. Calculation: 0.21 × cc. sediment = per cent. albumin by weight.

Roberts and Stolnikow take advantage of the fact that, with Heller's test, 0.0033% albumin will give a just perceptible "ring" in *exactly* two minutes. Earlier appearance indicates a stronger, and a later a weaker, concentration. A series of dilutions of the urine with 0.6% NaCl are made, and then one determines with which a "ring" appears at the end of just two minutes standing. I have used the U-tube mentioned above with this method, and found it satisfactory. Calculation: 0.0033 × dilution = per cent. albumin in urine. Example: 1–40 was the determined dilution; then 40 × 0.0033 = 0.13%.

Tsuchiya, using Esbach's albuminometer, employs a reagent made by dissolving 1.5 Gm. phosphotungstic acid in a mixture of 5 cc. concentrated HCl and 95 cc. 95% alcohol. It is generally considered more reliable than Esbach's reagent. Fill tube with urine to the U-mark, add reagent to R-mark, mix gently by inversion, let stand upright for twenty-four hours, and read height of precipitate. The urine

should be diluted if much albumin is present or if the specific gravity is high. The tube is graduated to read in grams of dry albumin per liter.

To estimate *globulin*, exactly neutralize the urine, filter, and add an equal volume of saturated neutral $(NH_4)_2SO_4$. The globulin is precipitated immediately, and is removed by filtration. The difference between the results of the above quantitative tests upon the original urine and the final filtrate roughly represents the amount of globulin.

Bence-Jones protein is characterized by its behavior with heat. Pour a few cubic centimeters of urine, acidified faintly with acetic acid if alkaline or neutral, into a test tube, and heat carefully immersed in a beaker of water, the temperature being taken by a thermometer the bulb of which is immersed in the urine. A positive result shows coagulation below 60°C., probably after the appearance of an opalescence below 50°C. This precipitate tends to disappear upon boiling, and to reappear upon cooling, but this feature may be obscured by serum albumin present. When precipitated by HNO_3, it behaves in the same way as upon boiling, while a precipitate of serum albumin will remain intact.

In pyelitis, nephritis, and cystitis, the urine often contains a substance called "*nucleoprotein*," which gives a positive reaction with Heller's test, and also the ordinary acetic acid and heat test. It is characterized by giving a turbidity after urine has been diluted with 3 volumes water and then acetic acid added until present in 0.2% strength, the reaction being especially well marked if salts have previously been removed from the urine by dialysis.

Glucose.—Reducing substances are normally present in urine to the extent of less than 0.2%, usually less than 0.1%, for a 24-hour specimen. The output varies during this period, that collected 3–4 hours after a heavy carbohydrate breakfast being most likely to show large amounts and that before breakfast least. Folin and Berglund claim that glucose is excreted only when the kidney "threshold" for sugar is passed. This threshold value for sugar varies with age and disease, being normally found at a blood sugar level of 160–180. We have had cases of diabetes mellitus with blood sugars of 350 and no abnormal glycosuria. Myers and Bailey reported a blood sugar of 1100 with only 0.5% sugar in urine. For the tests given below, any albumin present, especially if present in more than traces, should be removed (see p. 727).

Benedict's qualitative test is generally considered the most satisfactory of those requiring copper solutions, as it is less liable to give false positives and is 10 times as delicate as most of the others, it being said that 0.1% glucose gives precipitates of considerable bulk. Thus the test will be positive with urines having a high normal "sugar" content. The detection of such traces, however, is an advantage in that it calls attention to diabetics with high thresholds for sugar or who are in the "prediabetic" stage. Furthermore, unless one can account for such traces by reason of other causes, such as alimentary, pregnancy, anaesthesia, drugs, etc., a blood sugar estimation is indicated. We use the test as routine, and do not have a disturbing number of positives.

To about 5 cc. Benedict's reagent in a test tube, add a pebble or two to prevent bumping and 8–10 drops urine (not more) and mix. Boil *vigorously* and *carefully* for 1–2 minutes, and let cool spontaneously. A general turbidity, green, yellow, or red, the color depending upon the content, and due to a colloidal precipitate, is

positive. A clear solution, or a whitish or bluish turbidity due to precipitated urates, is negative. To prepare the reagent, dissolve, with the aid of heat, 173 Gm. sodium citrate and 100 Gm. anhydrous, or 200 Gm. crystallized, Na_2CO_3 in about 700 cc. H_2O, and filter if necessary. Add slowly, and with constant stirring, 17.3 Gm. $CuSO_4$ dissolved in about 100 cc. H_2O. Cool, and dilute to 1 liter. It will keep for years.

The *fermentation test* is said to demonstrate 0.05–0.1% sugar. It may, however, give false positives because yeast can form CO_2 from amino and other aliphatic acids, some of which are always present in urine. Faintly acidify the urine with tartaric acid, add to yeast in a mortar in the proportion of about 15 cc. to 1 gram, and rub until smooth. Place the mixture in a fermentation tube (Fig. 4), being careful not to include any air bubbles, and incubate for a few hours. If sugar is present, gas will collect in the closed end of tube. Controls are advisable—one of yeast and normal urine to eliminate the possibility of sugar being present in the yeast, and one of normal urine, yeast, and glucose to make certain that the yeast will produce gas.

The *phenylhydrazine test* is supposed to demonstrate the presence of 0.03–0.5% glucose, but the delicacy of the test with urine is such that the latter figure is more probably correct. Only positive results are significant, as results of application of this test are very inconsistent. Practically, it has no fallacy except that arising from the presence of levulose. Success is largely dependent upon the relation of the sugar to the reagents, the best proportions being approximately Sugar: phenylhydrazine: sodium acetate:: 1:2:3. Add a few drops concentrated lead acetate solution to 10 cc. urine, and filter. Add sufficient acetic acid to acidify, then 0.5–1.0 Gm. phenylhydrazine hydrochloride and about 2 Gm. sodium acetate. Shake well, heat in boiling water for one to two hours, replacing the fluid lost with water, and filter hot. Cool slowly and quietly in the water bath. Examine precipitate under the microscope. A positive result shows slender, yellow, needle-shaped crystals aggregated in fan-shapes or sheaf-shapes.

Benedict's quantitative method is quite satisfactory for clinical purposes. Place 25 cc. Benedict's *quantitative* reagent, equivalent to 50 mg. glucose, in a 500-cc. Pyrex Erlenmeyer flask, add 10–20 Gm. crystallized (or 5–10 Gm. anhydrous), Na_2CO_3, and a small quantity of powdered pumice stone or talcum, and boil until the carbonate is dissolved. Add the urine, diluted 1–10 unless sugar content is believed to be low, rapidly from a burette until a chalk-white precipitate forms and the blue color begins to fade, and then a few drops at a time until the last trace of *blue* disappears. Loss by evaporation must be replenished by addition of H_2O, and the mixture must be kept boiling vigorously. Calculation is simple, inasmuch as the urine added must have contained 50 mg. glucose. The reagent keeps indefinitely. To prepare it, dissolve, with the aid of heat, 200 Gm. of crystallized, or 100 Gm. anhydrous, Na_2CO_3, 200 Gm. sodium (or potassium) citrate, and 125 Gm. KCNS in sufficient H_2O to make about 800 cc., and filter if necessary. Add slowly, and with constant stirring, 18 Gm. crystallized $CuSO_4$ dissolved in about 100 cc. H_2O. Add 5 cc. 5% potassium ferrocyanide, cool, and dilute to 1 liter.

If a micro method is desired 5 cc. Benedict's quantitative solution may be used and the titration carried out in a 25 × 200 mm. Pyrex test tube. Add 2 Gm. Na_2CO_3 and a small pinch of talc proceeding as above. Calculation: 10 mg. glucose required to reduce 5 cc. Benedict's quantitative solution.

Roberts utilizes the loss in specific gravity as the result of fermentation for approximate quantitative estimation of glucose, and we have applied his principle as follows. Take the specific gravity of the urine, and then acidify and treat with yeast as described under the qualitative test above. Fill a cylinder exactly to the 50-cc. mark with this mixture, and incubate for 24 to 48 hours. Cool to the temperature at which the specific gravity was taken originally, replenish any loss of fluid by adding H_2O, filter, and again take the specific gravity. The difference between the two specific gravities, multiplied by 234, gives the percentage of sugar. One should determine the completeness of the fermentation by testing the filtrate for sugar.

Acetone Bodies.—These comprise acetone, diacetic acid and β-hydroxy-butyric acid. It is said that the kidneys do not excrete acetone but do diacetic acid, the latter changing into the former in the urine, which, when freshly passed, contains about 10 times as much diacetic acid as acetone. More acetone forms as the urine stands. The usual clinical tests for acetone are really delicate tests for diacetic acid. This fact, however, is unimportant inasmuch as the substances have the same significance. The tests will be more delicate and reliable if a distillate is used, the diacetic acid being changed, of course, to acetone. Faintly acidify more than 250 cc. urine with phosphoric acid, and distil, collecting the first 20 cc. of the distillate for use. It is to be noted that rubber connections may cause false positives. The urine should be as fresh as possible.

Gerhardt's test for diacetic acid, when strongly positive, usually indicates more than 0.05%. To about 5 cc. urine in a test tube, add 10% aqueous ferric chloride drop by drop until no further reddening occurs. A Bordeau-red color is positive, and will be more evident if the precipitate of ferric phosphate be removed by filtration. Many other substances, especially drugs, cause positive reactions, but may be differentiated by the fact that a red produced by diacetic acid disappears after boiling two to three minutes.

Lange, by methods accredited also to Taylor, tests for acetone bodies by adding 1–2 cc. 10% acetic acid and a small crystal of sodium nitroprusside to about 5 cc. urine in a test tube, shaking to dissolve the salt, and overlaying with concentrated NH_4OH. A purplish layer at surface of contact at once or upon standing is positive. The test is sensitive to 1 part of diacetic acid in 30,000, being much more delicate than Gerhardt's test; 1 part acetone in 2000 giving a faint color in about 20 minutes. That is, Lange's test is much more sensitive to diacetic acid than to acetone. For this reason *Rothera's test* which is more sensitive to acetone may be preferred: add one gram ammonium sulphate to 10 cc. urine and shake well. Then add 3 drops of a fresh concentrated aqueous solution of sodium nitroprusside and overlay with strong ammonia. The appearance of a reddish purple ring at line of contact is positive. Shades of brown in this test as in Lange's should be regarded as negative.

Hart's test detects 0.3% β-hydroxy-butyric acid. Dilute about 20 cc. urine in a beaker with an equal amount of H_2O, add a few drops of acetic acid, and reduce in volume to about 10 cc. by boiling. Add an equal amount of H_2O, mix, and place 10 cc. in each of two test tubes. To one tube, add 1 cc. hydrogen peroxide, warm gently, without boiling, for one minute, and cool. To contents of each tube, add 10 drops glacial acetic acid and 5–10 drops of a freshly prepared sodium nitroprusside solution, mix, overlay with concentrated NH_4OH and let stand three to four hours.

A positive result is a purplish layer in the tube treated with peroxide and none in the other. Creatinine may cause a yellowish-brown layer.

Indican usually results from decomposition of intestinal contents, but also accompanies protein putrefaction elsewhere in the body, such as occurs in abscesses, putrid bronchitis, etc. Obermayer treats about 10 cc. urine with 1 cc. Liq. plumbi subacetatis, and filters. Mix about 6 cc. of filtrate with an equal amount of reagent (0.1 Gm. ferric chloride in 50 cc. concentrated HCl), let stand for 5 minutes, and then mix gently with 2 cc. chloroform. Indican produces a blue color, the depth of which affords a rough index of the amount present.

Urobilin (for significance see p. 738 under Hepatic Function).—A large amount of urobilin gives the urine a dark brown or reddish color suggesting bile or blood and colors the foam produced when the urine is shaken in a test tube somewhat as does bile. The presence of small amounts of urobilin in urine may occur normally. Fluorescence with Schlesinger's test in urine which has been diluted 1–20 or more is considered pathological.

If bile be present, remove it by adding one-fifth volume 10% $CaCl_2$ and one-fifth volume of concentrated solution of Na_2CO_3 and filtering. Schlesinger's test is as follows: To 10 cc. urine add a few drops Lugol's solution (changes urobilinogen to urobilin), mix with an equal quantity of saturated alcoholic solution of zinc acetate and filter. Observe filtrate in a test tube for fluorescence utilizing sunlight and a black background. Use of a lens to concentrate light aids in detection of fluorescence.

Bile Pigments.—Tests for bile pigments in urine are by no means satisfactory. A yellowish foam produced upon shaking is quite reliable providing other causes of the coloration, such as urobilin, drugs, etc., be eliminated. *Rosenbach's test* is a modification of Gmelin's. The urine, acidified with HCl, is passed several times through filter paper, and the damp apex is then touched with a drop of yellow, commercial concentrated HNO_3. A positive result is a play of colors appearing in the following order: Green, the characteristic color, then blue, violet, red, and reddish-yellow. Antipyrin and indican interfere. Bile pigments may be precipitated, as given under urobilin or by the addition of lime water, and thus concentrated. Gmelin's test may then be applied to the precipitate collected on the filter.

Bile Acids.—Bile pigments and bile acids usually occur together in urine, and have the same significance. Oliver's test has a delicacy of more than 0.5%. Filter urine until clear, acidify if not already acid, and dilute with H_2O until specific gravity is less than 1.008. To 2 cc. of the diluted urine, add 5 cc. reagent, made by dissolving 8.33 Gm. peptone and 1.12 Gm. salicylic acid in 1 liter distilled H_2O containing 2 drops acetic acid, the reagent being filtered until transparent. A milky turbidity (if it disappears when shaken, it will reappear upon addition of more reagent) is positive.

Occult Blood.—The precautions of p. 720 should be applied, and alkaline urines faintly acidified with acetic acid. Donogány (see p. 721) secures a spectroscopic delicacy of about 1–8000 in addition to the color reaction by treating 10 cc. urine with 1 cc. each of fresh ammonium sulphide solution and pyridin (not old). A spectroscopic delicacy of about 1–1500 is given by coagulating 200–500 cc. urine by heat, egg albumin being added if necessary, acidifying, centrifuging, and using ethereal extract of sediment as per p. 720.

Diazo Reaction.—Add 1 part freshly prepared 0.5% sodium nitrite to 100 parts of a solution made by dissolving 1 Gm. sulphanilic acid in 10 cc. concentrated HCl and 200 cc. H_2O. To about 5 cc. of the mixture in a test tube, add an equal volume of urine, shake well, and quickly add 1–2 cc. strong NH_4OH. A pure shade of red, especially in the foam, is a positive reaction, any trace of yellow or orange denoting a negative result.

Formaldehyde in urine after administration of methenamine.—To inhibit bacterial growth, 1 part formaldehyde must be present in 5000 parts urine, a condition that is secured in perhaps only 48% of those taking methenamine as a genito-urinary antiseptic. If free formaldehyde is to be present the urine must be acid; hence, if necessary, dihydrogen (acid) sodium phosphate should be administered until the reaction is acid. Burnam's test is a practical index to efficacy of treatment. Remove any albumin by careful boiling of urine and filtration. To about 10 cc. urine at 37°C. in a test tube, add 3 drops each of 0.5% phenylhydrazine hydrochloride and 5% sodium nitroprusside. Run a few drops of 20% NaOH down wall of test tube, and allow it to diffuse through the mixture. A satisfactory result will be a deep purplish-blue color that changes rapidly to dark-green, lighter green, and finally pale-yellow. A reddish color changing to a light-yellow indicates insufficient formaldehyde.

I—KIDNEY FUNCTION

The ability of the kidney to excrete substances from the blood stream is frequently affected by disease, especially such as disturbs the kidney, and is usually diminished after the age of fifty years. Disease of the kidney, however, does not necessarily imply an inability to eliminate substances, that is to say, the functional lesion need not parallel the anatomical. Function, even when serious disease exists, may be normal, especially when the changes are of focal type. Moreover, impairment of renal function does not affect the excretion of different compounds to the same extent. It is well established that functions for chlorides and for urea are independent of each other. In any urine examination, then, one should restrict conclusions regarding impairment of excretory power to the substance under consideration. In this connection, we speak of the kidney "*threshold*" for a substance, i.e., the lowest concentration in which the substance must be present in the blood stream before the kidney will excrete it, at least in abnormal amounts. For chlorides this is quite definite at 562 for blood plasma; for sugar, it is about 160–180 for whole blood. The threshold is not necessarily absolute, but simply indicates that with less sugar, for instance, only the normal traces of reducing substances are passed. Chloride excretion begins only when the chloride concentration passes the threshold value, and the rate of elimination depends upon its excess. Other compounds, such as urea for example, may have no definite threshold value. Disease may affect the value, either by raising it and causing abnormal retention, or by lowering it and giving rise to depletion.

By combining the findings derived from blood chemistry and other tests of renal function, we are in a position intelligently to adjust the diet, etc., to the excretory powers of the kidney, always bearing in mind, however, that the nutritive needs of the body cannot be neglected. Renal function and acidosis determinations are

equally important in connection with many phases of *surgical practice.* The former is especially material in urology, notably in connection with the average prostatectomy. A few days' drainage of the bladder, restricted diet, or other treatment, may alleviate an impaired renal function due to obstruction or infective processes in the lower genito-urinary tract. Relief of an infective process in one kidney usually improves the functional capacity of the other when it has been decreased through systemic channels. Focal infections elsewhere may impair kidney function. A decrease in nonprotein nitrogen in response to treatment is favorable, and the maximum result will be obtained in less than one month of hospital treatment. Vesical neoplasms affect the urea function in the same manner as prostatic obstruction. It behooves the urologist, in the presence of impaired renal function, to proceed with caution when surgical procedures are contemplated. The phthalein test and the blood urea are considered suitable guides. The operative prognosis would be unfavorable, from the standpoint of renal function, with a phenolsuphonephthalein excretion less than 40% for two hours, less than 20% for the first hour, or an appearance time of more than 25 minutes; or a blood urea nitrogen of more than 25. Evidence of still greater impairment calls for preliminary treatment as indicated above. Fifty for blood urea nitrogen is usually deemed an absolute contraindication to immediate operation. The general prognosis is grave (except in acute conditions) with a low phthalein excretion plus a urea nitrogen of more than 30 that cannot be lowered by preliminary treatment. An associated increase in creatinine of the blood adds to the gravity. In acute conditions, the tests are of but little prognostic value.

Many methods are employed for the determination of kidney function, and their relative values are still debatable. Their multiplicity indicates that no single test is entirely satisfactory, and one should always apply two or more to the case before forming a conclusion. Chemical analysis of the blood, the phenolsulphonephthalein test, and Mosenthal's method are much used. Ambard, McLean, Van Slyke, and others have devised formulae to this end, based upon chloride, urea, or sugar excretion, which have many warm advocates as well as severe critics. A rough clinical comparison of the two kidneys may be obtained by determining the urea in specimens of urine simultaneously collected by the ureteral catheter. The urea concentration test of MacLean and de Wesselow also gives data of value. Many prefer a method that places the kidney under a strain. The urea concentration test above answers this purpose, as do also two other tests that are very simple and strongly recommended, viz., the water ingestion, and the water concentration.

Water ingestion test.—Drink 1500 cc. water on an empty stomach, and take nothing more by mouth until the test is finished. Collect the urine during the first six hours. Normally, at least 1200 cc. will be secured, and at least one specimen will have a specific gravity as low as 1.003.

Water concentration test.—Beginning in the morning on an empty stomach, the patient eats only his customary *solid* food for 24 hours, no water or beverages of any sort being consumed. His urine is collected, and the specific gravity of each sample determined. He must void at least before retiring, once during the night, and again in the morning at the end of the 24 hours. Normally, at least one specimen will have a specific gravity as high as 1.030.

Blood Chemistry.—It measures excretory function for normal metabolic products, and has the additional value of being an aid in diagnosis and prognosis and a

guide to treatment, especially dietetic. It has the disadvantage of entailing a possibly unfamiliar technique, and does not afford a comparison of the two kidneys.

The substances usually considered are the nitrogenous compounds (nonprotein nitrogen, urea nitrogen, uric acid, etc.), but retention of others (sugar, chlorides, cholesterol, etc.) are also of significance in this connection. Of the nitrogenous constituents, the kidney excretes creatinine most readily, urea next, and uric acid with the most difficulty. As a consequence, an impairment of function usually results first in the retention of uric acid, then urea, and, finally, creatinine. Owing to the relatively small amounts of uric acid and creatinine present, the nonprotein nitrogen, which includes the nitrogen in them as well as in other compounds, is affected very little by alterations in their amounts. The nonprotein nitrogen value is dependent chiefly upon the urea content. This fact is the basis of an intelligent interpretation of the findings. And it is said that urea excretion is normal until three-fourths to six-sevenths of the total functional capacity of the kidneys is lost. Nonprotein nitrogen increases only when about 60% has been lost, and retention is rare unless the phthalein excretion is less than 40% for two hours. Hence, an increase in urea nitrogen or nonprotein nitrogen means bilaterally impaired function. The urea and nonprotein nitrogen are so markedly affected by diet, especially among nephritics, that caution must be exercised when they are employed as indices of renal function. This has not been properly appreciated in the past, which probably accounts for much of the discredit cast upon blood chemistry in this connection. High-protein diets will increase the normal values for nonprotein nitrogen by about 5, and changes in diet are not reflected immediately in the nonprotein nitrogen, but only after a few days. Moreover, one must bear in mind that persistently high figures for nonprotein nitrogen despite diet do occur, and may indicate merely a high level of nitrogenous equilibrium instead of retention. Continuance of a restricted diet in such cases would be of doubtful value. The nonprotein nitrogen is inversely affected by fluid intake. The uric acid, since it is less exogenous in origin, and since an increase appears early in the course of retention, is perhaps the most delicate and the safest index. 3.5 may be considered the high normal value. Positive results are, however, more significant than negative findings in our experience, as about 20% of our cases have shown normal blood uric acid accompanying definitely increased urea nitrogen. In the absence of other nitrogenous retention, an abnormally high blood uric acid would mean either focal infection somewhere, gout, or impaired kidney function.

We have found the uric acid determination of much assistance in judging the significance of occasional casts and traces of albumin in urine—an increase speaking for an organic kidney lesion. On the usual restricted hospital diet, over 20 for urea nitrogen should be considered suggestive of impaired kidney function; over 75 speaks decisively for renal involvement and probable uraemia. Values for "creatinine" of over 4 do not occur without great impairment of renal function and probability of uraemia.

Phenolsulphonephthalein (Phthalein, or Red) Test.—This was developed by Rowntree and Geraghty, and its simplicity makes it very useful, especially to the isolated practitioner with limited laboratory facilities. It estimates only excretory ability at the moment for a foreign substance, is not considered quite as reliable as chemical analysis of the blood, and, of course, does not give the additional information

that the latter supplies. Also, in certain cases, complete collection of urine sample may be difficult. It is, however, of much value, gives positive results before urea retention appears, has no contraindications, and does compare the kidneys and evaluate each when combined with ureteral catheterization or use of a separator. Diminished excretion of the dye occurs only when about 50% of the kidney function is lost. Positive results are of more significance than negative. Values of more than 75% for two hours may be accompanied by diuresis, and Frank considers such a finding suggestive of renal disturbance with irritation if there is any corroborative evidence.

The technique comprises administration of the dye, determination of the interval before it appears in the urine, and the amount then excreted during definite periods. The dye is employed in solution, and is conveniently purchased already sterilized

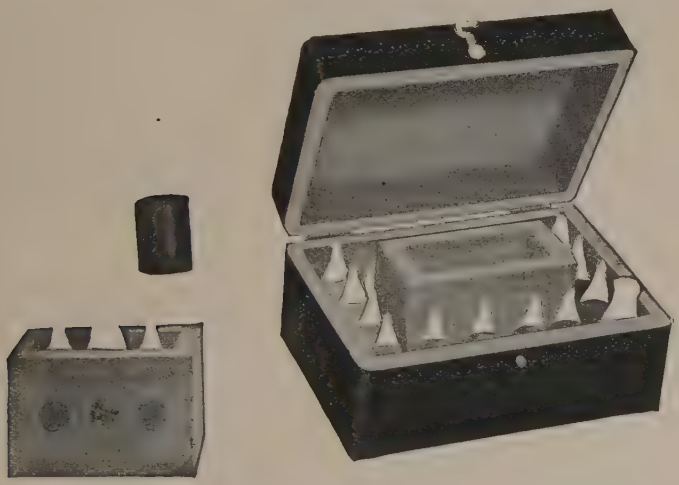

FIG. 210.—Dunning colorimeter.

in ampules, each containing slightly more than 1 cc. of a solution of its monosodium salt of the strength of 6 mg. per cc. The patient drinks 200–400 cc. water, and 6 mg. of the dye is injected intramuscularly (lumbar muscles), or intravenously, twenty minutes later. The bladder is immediately emptied, and the urine discarded. The succeeding portions of urine may be collected by voiding, but it is more accurate to catheterize the bladder or ureters, and catheterization is practically a necessity for determination of the "*appearance time.*"

The appearance time is the interval of time elapsing between injection of dye and its appearance in the urine. It is determined by allowing the urine to drip from the catheter into a receiver containing a drop of 10% NaOH. The first traces of the dye will cause a pink color.

The time interval chosen for calculating excretion is then computed from the instant of this appearance. There is considerable diversity in practice as regards

this time interval, and it would seem that shorter intervals and quicker results are gaining in preference, as well as being considered equal in value to longer periods.

The percentage excretion of the dye is now measured in each sample of the collected urine. One may employ the simple and inexpensive colorimeter of Dunning (Fig. 210), but it is better to prepare a fresh standard, as the colors of such permanent ones tend to fade. We also prefer to follow the suggestion of Cabot, and use standards in test tubes, rather than in the ordinary colorimeter, because off-colors frequently occur and a satisfactory match of unknown and standard is then impossible in the latter instrument.

Prepare a 100% standard by diluting 1 cc. of the above phenolsulphonephthalein solution to 1000 cc. with water. Using test tubes and rack such as those for pH determinations (see p. 692), measure into the test tubes portions of this standard, each tube receiving 0.5 cc. less than the preceding one. A series from 6 to 1.5 cc. is satisfactory, and gives standards representing 60% to 15% with 5% intervals. Add 1 drop 10% NaOH to each tube, fill to 10-cc. mark with water, and mix. Dilute the specimen of urine to some convenient volume that will permit a color match in the standard series, adding 10% NaOH so that it will be present in the final volume in the proportion of roughly 5 cc. per liter. Place some of this diluted urine in another test tube, compare it with the standard series, and estimate its percentage strength, interpolating if necessary. To calculate the per cent. of dye excreted in the sample, multiply the determined percentage by the total volume (in cc.) to which it was diluted, and divide by 1000.

An accurate determination demands "backing" of the tubes (see p. 693). For this purpose, place behind the standard tube a tube of dye-free urine (preferably that voided by the patient before test sample is taken) that has been diluted with water to the same extent as the unknown. Use a tube of water behind the unknown. Centrifuge the unknown before use if it is not clear.

After intravenous injection, the normal appearance time is 4–6 minutes, and the normal elimination is 35–40% in 15 minutes, 50–65% for 30 minutes and 65–80% for 60 minutes; or, for the first 30 minutes, it may be stated as about 1% per minute from each kidney.

After intramuscular injection, the normal appearance time is nearer 10 minutes, and the normal elimination is 30–40% in 30 minutes, about 50% (40–60) for 60 minutes, and about 80% (50–85) for 2 hours, or 20–25% during the second hour. If the appearance time is not determined it is customary to allow for it, collecting the first hour's specimen at 70 minutes after injection of dye and the second hour's 60 minutes later.

Impairment of kidney function, of course, increases appearance time and lessens excretion, serious cases not unusually excreting less than 1% during two hours.

When the question of the kidney involved arises, the urine must be taken by ureteral catheterization or by a separator.

Mosenthal's Test.—Mosenthal's test is a refinement of previous work that endeavored to estimate renal function by measuring the ability of the kidneys to concentrate and excrete substances that occur normally in the blood stream, as water, chlorides, and nitrogen. As originally described, it involved the use of a definite diet that he now terms the "high protein diet." A "low protein diet" and the diet normal for the patient were later tested, and he found that the essential

facts held with all. The result has been a simplification of technique that enables wider application and its use for ambulant cases in ordinary private practice. The test is probably more sensitive for impaired kidney function than either the blood urea or phthalein test.

At 8 A.M., let patient void and discard urine, and eat breakfast. Urine is to be voided and collected only at 10 A.M., 12 Noon, 2, 4, 7, and 10 P.M., and at 8 A.M. on following morning, this last sample being the night urine. Luncheon may be taken at 1 P.M. and dinner at 7 P.M., *three hours before last evening collection of urine.* The meals are those to which the patient is accustomed, and a record of everything consumed is kept in round figures in order that a basis for criticism of diet may be at hand. No food or drink is to be taken except at meals. Determine volume and specific gravity of each sample, and the twenty-four hour excretion of NaCl and nitrogen. Mosenthal states that the results obtained by dividing the urea value obtained by the hypobromite test by 2.14 (the N factor) is a sufficiently accurate expression of the total nitrogen value for the purposes of the test.

As normals, he gives 1.020 (or 1.018 with the "high protein diet") or over in any specimen, as a maximal specific gravity, the extreme values of the different samples to show a variation of 0.009 or more, and a night urine sample of 750 cc. or less. By "fixation of specific gravity," is meant a variation between extremes of less than 0.009. A normal maximal specific gravity indicates that the kidney can concentrate the urine satisfactorily, providing the twenty-four hour amount of urine is adequate. A high specific gravity with oliguria occurs only in passive congestion of the kidney, and in acute, subacute, or chronic diffuse nephritis, conditions that also show a markedly diminished NaCl excretion together with considerable albuminuria and oedema. Long life is often possible providing a low specific gravity is compensated by polyuria, as in diabetes insipidus and a few cases of chronic nephritis. A high, fixed specific gravity of about 1.020 may occur in normal individuals as the result of insufficient fluid intake, or may be the result of diseases characterized by oedema and oliguria, especially myocardial insufficiency and acute or chronic nephritis. A low, fixed specific gravity is a danger signal which is found in many widely varying conditions, as diabetes insipidus, chronic nephritis, marked anaemia, during the elimination of oedema, cystitis, pyelitis, polycystic kidney, prostatic hypertrophy, urethral stricture and paralysis of the bladder, such as occurs in tabes dorsalis, tumor of the cord, etc. Such cases may do well as long as there is a compensatory polyuria.

A nocturnal polyuria indicates an overworked kidney, and the strain may result in functional damage. Over 400 cc. should be considered somewhat suspicious of renal insufficiency. In nephritis, it may be improved by curtailing the food intake.

NaCl is being ingested in unnecessary amount if the twenty-four hour excretion exceeds 5 Gm. A very low excretion, together with oedema, indicates an inadequate elimination, and is then not a criterion of the diet.

If 5 or 6 Gm. nitrogen are eliminated in the urine every twenty-four hours, there is sufficient protein in the food to maintain the individual's health and strength, provided the diet contains a considerable amount of starch. Restriction of proteins should be guided by the nature of the disease.

Thus the test supplies information useful in diagnosis and prognosis, is an early index of renal function, and serves as a guide to diet. Chemical analysis of the blood, especially of the nitrogenous constituents, will furnish further details as to the manner

in which the intake of nitrogenous foods and NaCl should be varied. Conclusions should be based upon more than a single test.

Urea concentration test.—This, a simple test, is generally considered to be reliable, more delicate than the blood urea, and without contraindications (except effects of possible nausea). Patient voids urine, and then takes by mouth 15 Gm. urea dissolved in 100 cc. water, and flavored, if desired, with Tr. aurantii. At end of one hour, void and measure urine. At end of second hour, void, measure urine, and determine percentage of urea in this specimen. A urea concentration of 2% or over in the second specimen indicates a satisfactory function. Less than 2% means impairment that is directly proportional to the departure from the normal value. Values of less than 1% are usual in uraemia. A total urine of more than 300 cc. for the two hours is due to diuresis, and a low urea concentration then would not have the same significance.

J—LIVER FUNCTION

The liver performs or helps to perform a number of different and apparently unrelated functions. It, 1. stores carbohydrate as glycogen; 2. helps to maintain the blood sugar level; 3. excretes bile pigment; 4. excretes cholesterol and bile salts (synthesis in question); 5. is the important seat of intermediary nitrogen metabolism; 6. detoxifies many substances; 7. helps to remove particulate matter and bacteria from the blood; 8. aids in the production of clot forming elements of the blood; 9. excretes a number of foreign chemical substances such as dyes in the bile; 10. excretes absorbed urobilin (hydrobilirubin). With this multiplicity of function it is obviously impossible to obtain a single test that will cover all. As it is very probable that the different functions are variously affected in different cases of liver damage or malfunction, the result of an individual test must be interpreted with care. Many of the tests devised have failed to give reliable data of clinical value or have not been found to be based on any known physiologic function of the liver. In many cases it has been found very difficult to separate the specific function of the liver from the activity of the organism as a whole, for while the liver plays the major role in many physiologic processes its function may be only a part of the process in which other organs are necessarily involved. Hepatic insufficiency with reference to some functions is concomitant with death, the liver steadily maintaining its activity to the last. Mann reports that many experimental animals have died apparently from the failure of some subtle function of the liver as yet unknown.

The liver function tests which appear to be of value are the dye excretion tests of Rosenthal and Rowntree (phenoltetrachlorphthalein and phenoltetrabromphthalein) which measure conveniently and accurately the ability of the liver to excrete that class of substances; the Icterus Index, a method which estimates the icteric tint of serum by comparison with an arbitrary potassium dichromate ($K_2Cr_2O_7$, 1 to 10,000) standard; and the Van den Bergh reaction which measures serum bilirubin in milligrams per liter (quantitative or indirect reaction) and differentiates obstructive jaundice from that of haemolytic origin (qualitative or direct reaction). The liver through its endothelial cells, forms only a portion of the bilirubin that is derived from the constantly liberated haemoglobin and which it alone excretes. The reticulo-endothelial cells through the body are probably the agents which are cap-

able of forming bilirubin, as Mann has shown, quite independently of the liver. So that in jaundice the liver may be directly or indirectly, although not necessarily entirely at fault. Accurate methods for the estimation of serum bilirubin bring to light cases of latent jaundice and disclose the presence, intensity and trend of an icterus in a manner impossible from the examination of sclera, skin or urine. Accurate estimation of bilirubinaemia and aid in determining the source of the pigment should prove of great value to the surgeon as well as to the clinician.

The site of *urobilin* formation (urobilin regarded as isomeric with hydrobilirubin) is in the intestinal tract from bilirubin. The only exception is when the biliary tract is infected. Part of this pigment is absorbed, carried to the liver and excreted in the bile. Small amounts may appear in the urine normally (see p. 731). Urobilin may be present in the urine in abnormal quantities in cases of excessive red blood cell destruction (excessive bilirubin formed from the liberated haemoglobin), in cases of biliary tract infection in which hydrobilirubin is formed by bacterial action in situ, in cases of hepatic insufficiency, or due to a combination of the above causes. Thus the import of pathological urobilinuria rests upon its pathogenesis.

Widal has suggested that the liver detoxifies protein cleavage products which come to it during digestion. He notes that when this "proteopexic" activity is interfered with by hepatic insufficiency these toxic products produce a leukopenia or "haemoclastic crisis" and a fall in blood pressure in contrast to the leukocytic rise which normally occurs during digestion. He produces this "*haemoclastic crisis*" by administration of 200 cc. of milk to the fasting subject and observes the maximal effect to occur in about 45 minutes. The diagnostic value of this test has as yet not been established.

A *levulose tolerance test* as a clinical test for hepatic glycogenic function has claimed some attention but its value is still debatable.

Mann states that there is definite experimental basis for the elaboration of a test for glycogen mobilization. His experimental evidence also indicates that, in dogs, destruction of uric acid is performed entirely by the liver and that this function is the first to be interfered with when liver insufficiency is surgically produced. It is probable, he believes, that the amount of uric acid excreted in the urine of persons on a diet high in purins may serve as an index of hepatic efficiency. The value of this test in man has yet to be proved.

Icterus Index (Meulengracht method modified as given by Bernheim).—Draw 10 cc. of blood by venipuncture into a centrifuge tube and allow to clot. Separate serum by centrifuging and pipette off. Compare in a colorimeter with an arbitrary standard, 1 to 10,000 potassium dichromate ($K_2Cr_2O_7$, 0.050 Gm. to 500 cc. dist. water). The unknown (serum) is read in a colorimeter against the standard set at 15. Divide the reading at which the unknown matches the standard into the reading of the standard. This quotient multiplied by the dilution of the unknown (necessary when serum is deeply colored) equals the index. When dilution is necessary use 0.9% NaCl.

$$\frac{\text{Standard}}{\text{Unknown}} \times \text{Dilution} = \text{Index.}$$

The normal range is given as 4 to 6, latent jaundice 7 to 16, and clinical jaundice from 17 up.

Other pigments such as carotin may color the serum and the slight haemolysis, which often occurs before the serum is separated from the clot, makes comparison difficult, and to the degree that the misleading color is present, inaccurate. This is especially noticeable in the lower ranges of color (normals, latent, and early clinical icterus) where accuracy means much.

Bromsulphalein Dye Excretion Test (Phenol tetrabromphthalein sodium sulphonate).—The technique given by Rosenthal is as follows: Weigh patient and calculate dosage on basis of 2 mg. bromsulphalein per kilogram body weight. Weight in pounds divided by 55 gives the exact quantity in cubic centimeters of the 5% solution to be injected. Measure bromsulphalein by aspirating sterile solution from ampule into sterile 5 cc. syringe. Inject slowly into an arm vein. Injection should take at least one minute. Avoid infiltration of dye outside vein. Thirty minutes after injection draw 5 cc. blood by venipuncture from opposite arm. Place in a dry centrifuge tube. In cases of early liver disease it may be advisable to obtain also a sample 5 minutes after injection.

Separate serum and pipette into two small test tubes. Add to one, 1 or 2 drops NaOH 10%, to bring out the color of the dye, and to the other add a drop of 5% HCl, to clear the serum of any haemolysis. Estimate the amount of dye present by direct comparison with a series of standards. Place the tube of clear acidified serum in front of the standard and back the alkalized tube of serum by a similar tube containing water, in a suitable comparator box.

Interpretation.—Normals are as follows: Five minutes after injection from 20 to 50 per cent. of the dye is present in the serum, with an average of 35 per cent. In 30 minutes after injection the dye is entirely gone or only a trace is present, too small to estimate. The amount of dye remaining in the serum at the end of 30 minutes may thus be taken as the numerical measure of the lost hepatic function. In normal cases the dye appears in the urine in negligible quantities ranging from none at all to 0.5% of the amount injected.

Preparation of standards.—Add 4 mg. bromsulphalein to 100 cc. distilled water alkalized with 0.25 cc. NaOH 10%. This is the 100 per cent. standard. By proper dilutions with similarly alkalized water prepare 10 standards ranging from 10 to 100 per cent. Seal in small test tubes. No deterioration occurs for several months if stored in the dark.

Phenoltetrachlorphthalein Dye Excretion Test.—The phenoltetrachlorphthalein test (of Rowntree and Rosenthal) has been used for some time. It was originally carried out by an estimation of the amount excreted in the 48-hour faeces. This dye is somewhat irritant and occasional reactions have been reported from its use and as it is necessary to use a dose of 5 mg. per kilogram body weight, many have considered more advantageous the use of bromsulphalein, which can be used in doses of 2 mg. per kilogram and is apparently not productive of reactions.

The technique is as follows. The sterile 5% aqueous solution may be freshly prepared or obtained in ampules. The dose is 5 mg. per kilogram body weight or 0.1 cc. of the 5% solution per kilogram. The calculated dose is given in 100 cc. sterile physiological saline solution by intravenous injection. Care should be taken to avoid infiltration outside the vein and the needle washed out with sterile saline solution. Fifteen minutes and one hour after the injection specimens of blood are removed from the vein in the opposite arm, allowed to clot, centrifuged and the serum removed.

Pipette serum into two small test tubes, add to one a drop of NaOH 10%, to bring out the color of the dye and to the other a drop of 5% HCl, to clear the serum of any slight haemolysis and compare as described under bromsulphalein.

Preparation of standards.—Add 10 mg. phenoltetrachlorphthalein to 100 cc. distilled water alkalized with 0.25 cc. NaOH 10% (0.2 cc. of the 5% solution contains 10 mg.). This is the 100% standard. By proper dilutions with similarly alkalized water standards of any desired strength may be obtained.

Interpretation.—In normal cases from 2 to 6% of the dye remains in the fifteen minute specimen and none in the one hour specimen.

Van den Bergh Reaction (*Modified*).—Van den Bergh developed his reaction by applying the Ehrlich diazo-reaction to sera containing bilirubin. The reaction depends upon the development of a red azo dye (azobilirubin), when an acid solution of a diazonium salt is added to a solution containing bilirubin. Van den Bergh determined that pure bilirubin in a dilution of 0.7 mg. per liter gave a positive reaction and that biliverdin and other substances in the serum did not.

Technique of the method.—(Reagents must be of best quality and reasonably fresh.) Draw 6 cc. blood by venipuncture into a dry centrifuge tube and allow to clot. Separate serum, by centrifuging if necessary, and pipette off. The diazo-reagent which must be made up fresh just before use is a mixture of two solutions:

Solution A.—Sulphanilic acid	1.0 Gm.
Concentrated HCl	15.0 cc.
Distilled water q.s.	1000.0 cc.
Solution B.—Sodium nitrite	0.5 Gm.
Distilled water q.s.	100.0 cc.

To prepare fresh reagent mix 25 cc. of solution A with 0.75 cc. of solution B.

The qualitative or direct reaction.—Place 0.25 cc. serum in each of three small test tubes. To tube No. 1 add 0.2 cc. water. To tube No. 3, 0.2 cc. diazo reagent (fresh). After waiting five minutes for reaction to become complete in control tube No. 3, add 0.2 cc. diazo reagent to tube No. 2. Watch and time development of any reaction. Prompt or immediate reaction begins before 30 seconds have elapsed. Comparison with serum control, tube No. 1, and completed reaction control, tube No. 3, will aid in detection of color.

The quantitative test or indirect reaction.—To 1 cc. serum in a 15 cc. graduated centrifuge tube add 0.5 cc. diazo reagent (freshly prepared as above). After a minute or two add 2.5 cc. 95% alcohol and 1.0 cc. saturated solution of ammonium sulphate $(NH_4)_2SO_4$. Mix well with a stirring rod after each addition and finally centrifuge.

The diazo-reagent is added before the alcohol to allow "coupling" to take place. By this method very little, if any, bilirubin is carried down with the precipitated protein, as the azobilirubin is very soluble in alcohol, while bilirubin is less so and is carried down with the precipitate in relatively large amount if the reagents are added in the reverse order. The color of the supernatant fluid will vary from a faint pink, as in normal serum, to a deep violet, depending on the amount of bilirubin present. The quantity of supernatant fluid is read on the graduations of the centrifuge tube and the dilution of the bilirubin contained in the cubic centimeter

of serum used is thus directly obtained. The quantity of bilirubin present in the serum (1 cc.) is now, as azobilirubin, entirely in alcoholic solution. As the color of the standard represents a bilirubin concentration of 5 mg. per liter the calculation is:

$$\frac{\text{Standard}}{\text{Unknown}} \times \text{dilution of unknown} \times 5 = \text{mg. bilirubin per liter of serum (using a plunger type of colorimeter).}$$

or

$$\frac{\text{Unknown}}{\text{Standard}} \times \text{dilution of unknown} \times 5 = \text{mg. bilirubin per liter of serum (using a dilution type of colorimeter).}$$

Note.—Dilution of unknown is read from supernatant alcoholic solution in graduated centrifuge tube.

Standard for the quantitative reaction:

Solution 1.—Ammonium ferric alum	0.1508	Gm.
Concentrated HCl	50.0	cc.
Distilled water q.s.	100.0	cc.
Solution 2.—Of solution 1	10.0	cc.
Concentrated HCl	25.0	cc.
Distilled water q.s.	250.0	cc.
Standard which is made fresh daily.		
Of solution 2	3.0	cc.
10% ammonium or 20% potassium sulphocyanate solution	3.0	cc.
Ether	12.0	cc.

Shake thoroughly. The ether extracts the color from the solution and forms a supernatant layer which may be used in colorimetric comparison. The standard matches in color a dilution of 5 mg. per liter of bilirubin.

By the use of cobaltous sulphate a permanent aqueous standard may be made which avoids many of the errors and difficulties inherent in the ether standard. Use 2.161 Gm. anhydrous cobaltous sulphate to 100 cc. water. This standard also represents the color given by 5 mg. bilirubin per liter. If unable to obtain the anhydrous salt use the crystalline cobaltous sulphate ($CoSO_4.7H_2O$) and make up by comparison with the ether standard as follows: Make an aqueous solution somewhat deeper in color than the ether standard, compare in colorimeter, and dilute as indicated to match the color in the ether standard. This solution keeps well in the dark. We have found that the addition of 0.5 cc. H_2SO_4 per 100 cc. does not change the color and the solution keeps thus indefinitely.

Interpretation.—The normal range of bilirubin is given as 1 to 3 mg. per liter, latent jaundice from 4 to 20, and clinical icterus from 20 up. If reported in units, one unit equals 5 mg. bilirubin per liter.

There are three possible results in the direct reaction: Immediate or prompt, beginning promptly after the reagent has been added and rapidly reaching its maximum color. Delayed, beginning after 30 seconds. These reactions develop

slowly, many not reaching their maximum until 30 minutes or more have elapsed. Negative, no color develops in 30 minutes.

The prompt direct reaction is given by the bilirubin in the obstructive type of jaundice. Delayed or negative reactions may be obtained in both normal sera and those from cases of non-obstructive or hemolytic jaundice. It was observed that reactions in sera from cases of jaundice, clinically of the toxic and infective type, were slow in arriving at their maximum color and the term "biphasic" was used to describe the reactions in which the maximum color did not develop before 30 seconds. We found (as did others) that reactions with sera from cases of obstructive jaundice often failed to reach their maximum before two minutes or more had elapsed. For this reason and until the significance of the completed reaction time is more clearly defined we have preferred to drop the designation "biphasic" and consider all reactions which begin before the thirty second limit as "prompt," with a notation as to the time the reaction began and was complete.

Observations of Rosenthal indicate that the presence of bile salts may play an important role in the availability of bile pigments for excretion, or reaction in vitro. His work indicates, he says, that bilirubin circulates in the blood firmly bound to the proteins and thus cannot be excreted by the kidneys, but is readily split from this adsorption compound in the liver by means of the bile salts. Blankenhorn does not agree with Rosenthal's bile salt theory, just mentioned. He states that bilirubin in simple solution gives the direct reaction while bilirubin in protein combination gives the indirect reaction. He believes that the rate of production governs the form in which bilirubin is present, that is, the pigment produced rapidly and rapidly poured into the circulation will be in simple solution and give the direct reaction and that produced slowly will form protein combinations and give the indirect Van den Bergh reaction.

K—CHEMICAL EXAMINATION OF THE STOMACH CONTENTS

We prefer the Rehfuss tube for collection of samples. The patient should take no fluids after the evening meal, which should include 2 or 3 prunes or several raisins. The stomach tube is passed in the morning before breakfast. While saying "ah," the metal tip is dropped behind the tongue and swallowed until 50 cm. (about 40 cm. is distance to cardia) is behind the teeth. Deep breathing minimizes nausea. It may be necessary to give a few cubic centimeters (measured) of water with the tube. Saliva must be expectorated, and none swallowed except with the test meal, and it is better not to brush the teeth before the test. Withdraw the residuum without strong suction while patient, breathing deeply, assumes successively the upright, recumbent, prone, knee-chest, and each lateral position. Administer the Ewald test meal, consisting of 35 Gm. toast with 240 cc. H_2O or tea, with the tube in place, and enjoin thorough mastication. Withdraw 6–8 cc. of gastric contents every fifteen minutes, clearing tube after each sample by injection of small amount of air, until no more food remains in stomach. This may be determined by straining the sample and testing for starch with iodine solution, and finally checking by lavage with 250 cc. H_2O.

Those who prefer the analysis of a single specimen of stomach contents, aspirated one hour after the test meal, will be interested in the findings of Fitz (see p. 625). With his routine, care must be exercised to secure the total contents.

The routine procedure includes the gross inspection for volume, chyme, mucus, bile, and any abnormality; the chemical examination for lactic acid, free acidity, total acidity, and blood; and the microscopic examination of the residuum. In the occult blood tests, observe the same precautions as when dealing with faeces (p. 720), and, as material, use mixed, unfiltered contents. For the other chemical determinations, it is customary to use a filtrate, the transparency of which may be improved by the use of wet filter paper, discarding the first few drops of filtrate.

Lactic Acid.—Place 5–10 cc. filtrate in a large test tube, add 0.5 cc. N/1 HCl and about 10 cc. ether; mix cautiously for three to four minutes. Remove the clear ether layer, and add ferric chloride solution to it a little at a time with shaking until

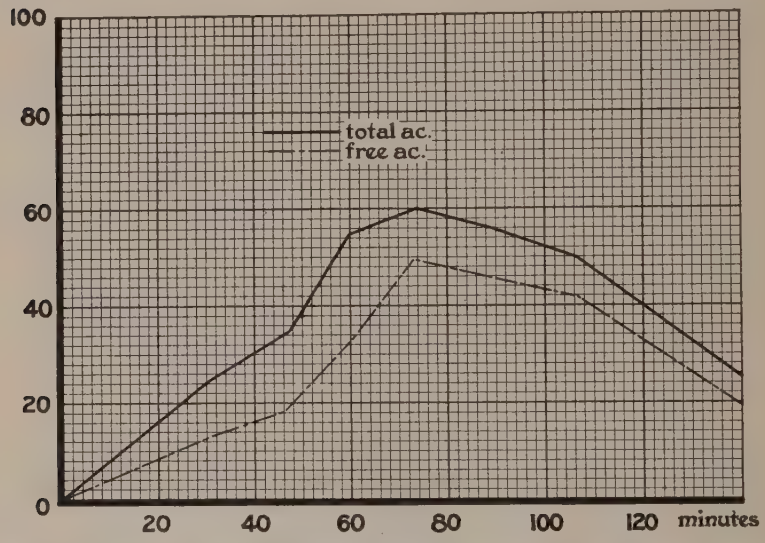

FIG. 211.—Acidity curves of normal human stomach. (*From Hawk's Practical Physiological Chemistry.*)

the maximum color is produced. A yellowish-green color is positive, and its depth, as well as the amount of $FeCl_3$ added, gives a rough index of the amount of acid present. Old, deeply-colored $FeCl_3$ solutions are unsatisfactory, and should be rendered pale by the addition of 1 cc. concentrated HCl per 5 cc. of 10% aqueous $FeCl_3$. For the test, dilute this $FeCl_3$ solution with about fifty parts of H_2O.

Free Acids.—In the absence of lactic acid, this practically means HCl. Titrate 10 cc. filtrate with N/10 NaOH, using 2–3 drops Töpfer's reagent (0.5% dimethyl-amino-azo-benzol in 95% alcohol) as indicator. A pure yellow is the end-point. Express the result as "degrees acidity" (cc. N/10 alkali necessary for 100 cc. filtrate). With this indicator, HCl gives a deep cherry-red color, while notable amounts of organic acids may produce an orange color. For the determination of free HCl, many recommend the use of thymol blue instead of Töpfer's reagent,

titrating to a pure yellow as the end-point, because results with Töpfer's are too high and thymol blue is less affected by organic acids, these producing comparable red shades only in strengths of at least about N/10. To determine free HCl in the presence of organic acids, another method is necessary, and one may employ Boas' reagent (5 Gm. resorcinol and 3 Gm. cane sugar in 100 cc. 50% alcohol) or Günzberg's reagent (2 Gm. phloroglucinol and 1 Gm. vanillin in 100 cc. 95% alcohol). One to two drops are evaporated to dryness in a porcelain evaporating dish on a water bath; while on the bath, the film of reagent is touched with a tiny drop of the filtrate; HCl causes a red color, while organic acids do not. The cessation of this reaction will serve as an end-point for the titration.

Total Acidity.—This comprises that due to free acids, combined acid and acid salts. In testing, one uses 2–3 drops phenolphthalein solution, 1% in 95% alcohol, as an indicator, the end-point being the first faint pink color. A new sample of filtrate may be taken, or the indicator added and the titration continued after the free acid has been determined. The total acidity then includes, of course, the free acidity already determined, and is expressed in degrees acidity.

Pepsin.—This is conveniently measured by means of Mett's tubes. These are prepared by filling capillary glass tubes of 1–2 mm. internal diameter with white of egg, plugging one end with a bread crumb, and coagulating in water at 97–98°C. for five minutes. They should be stored under water in the ice box.

Place two tubes, about 2 cm. long and full to the ends, in a test tube with some gastric filtrate diluted with 15 vol. N/20 HCl, and incubate at 37.5°C. for 24 hours. Measure the millimeters of the albumin column digested, average the findings, square the average, and multiply by 16. This gives the number of Mett's units of peptic activity of the gastric contents, and the normal is 8–100.

Trypsin.—This may be determined by Spencer's method. In a series of small test tubes, place 0.5 cc. of various dilutions of gastric filtrate with H_2O—pure filtrate in #1, 50% in #2, 25% in #3, etc., these dilutions then being 1/1, 1/2, 1/4, etc., respectively. Add 1 drop of 1% phenolphthalein in 95% alcohol to each, and then 2% $NaHCO_3$, until a light-pink color appears. In each tube, place 0.5 cc. casein solution made by dissolving 0.4 Gm. casein in 40 cc. N/10 NaOH, adding 130 cc. distilled H_2O, and then 30 cc. N/10 HCl. This casein solution will keep about one week if chloroform is added as a preservative. Incubate for five hours at 40°C. Then add to each tube an equal number of drops of 1% acetic acid in 50% alcohol. Record result as the figure of the greatest dilution with which no turbidity is produced, e.g., if tubes #1 and #2 are clear and #3 is turbid, the result is recorded as 2.

Protein.—Clarke and Rehfuss slightly modify the Wolff-Junghans' test. The residuum must be removed before test meal is given. In a series of six test tubes, place 5 cc. of various dilutions of gastric filtrate with H_2O—10%, 5%, 2.5%, etc. Stratify with 1 cc. reagent, consisting of 0.3 Gm. phosphotungstic acid, 1 cc. concentrated HCl, 20 cc. 95% alcohol, and distilled H_2O to make 100 cc. Read immediately against a dark background. Record results as the greatest dilution showing a turbid "ring" at plane of contact, e.g., a ring in the first tube but none in the second, would be reported as 1–10; in the third but not in the fourth as 1–30, etc. Normally, the protein curve, using the dilutions as ordinates, parallels the acidity curve, with no "ring" showing after the 1–40 dilution. Positive results with greater dilutions indicate abnormal amounts of albumin.

L—CHEMICAL EXAMINATION OF THE DUODENAL CONTENTS

We pass the tube as for gastric contents, remove any gastric residuum, and then let the patient swallow the tube until 55 cm., the average distance to pyloric orifice, is behind the teeth. Then administer a small glass of water, and have patient lie upon right side and make slow swallowing movements until the 75-cm. mark is reached. Flexing the knees, slightly elevating the hips, and other variations of position may sometimes be necessary. Ordinarily the tube will enter the duodenum within twenty to forty minutes. The position of the tip can be checked by noting the character of the fluid aspirated, by fluoroscopy, or by rapid aspiration which will collapse the thin rubber tubing when it is in the duodenum. A test meal may then be given to determine the duodenal response, Einhorn suggesting for this purpose a cube of bouillon in a cup of hot water. Siphon off the duodenal contents if possible, changing the receiving vessel whenever the gross appearance of the fluid changes, or the contents may be withdrawn fractionally as for gastric contents. Bile flow can be stimulated by injecting through the tube 5–50 cc. diluted HCl, 1 cc. U.S.P. Acidum Hydrochloricum in 200 cc. H_2O.

The examination should be made immediately, as the characteristics of the fluid change rapidly. The routine may comprise gross and microscopic features and chemical tests for reaction, ferments, and blood; possibly, bacterial flora also. Prior to testing for ferments, fluid should be made 8° alkaline to phenolphthalein with NaOH.

Reaction.—Titrate as for gastric contents, using N/10 NaOH or HCl as indicated. Einhorn gives the normal as 15–40° alkaline to methyl orange. We find it to range from neutral to 20° acid to phenolphthalein.

Trypsin.—We employ Gross' method in the following manner: In a series of six small test tubes, place 1.0, 0.5, 0.25, 0.1, 0.05 and 0.01 cc. alkalinized duodenal contents. To each add 2.5 cc. 0.1% casein in 0.1% Na_2CO_3, and mix. Add a 7th tube for control, containing only casein solution. Digest in water bath at 38°C. for fifteen minutes. Add a few cubic centimeters cold H_2O; then acidify each tube with dilute acetic acid, and mix. Note least amount of duodenal contents producing complete digestion indicated by transparency of the mixture, and report as number of cubic centimeters of casein solution that 1 cc. duodenal contents will digest. If this is the tube containing 1 cc. duodenal contents, report would be 2.5; if one containing 0.1 cc., 25, etc. We consider the normal to be 25–50.

Amylase (Amylopsin).—We apply Wohlgemuth's method in the same manner as in the previous test, using 1% soluble starch solution instead of casein, and digesting for thirty minutes. Test for starch with a few drops N/20 iodine in each tube. The tube with the least amount of duodenal contents, showing no blue color when mixed, is determined, and the result calculated as for trypsin. We consider the normal to be 2.5–5.0.

Lipase (Steapsin).—Place 1 cc. alkalinized duodenal contents in each of two large test tubes. Boil contents of one, and cool. To each tube, then add 1 cc. ethyl butyrate, 10 cc. H_2O, and 1 cc. toluene. Shake well, and incubate at 37°C. for twenty-four hours, shaking several times in the interval. Then titrate acidity, using N/10 NaOH and phenolphthalein. The difference (in cubic centimeters) between the results of the two titrations represents the lipase. We consider the normal to be 0.2–2.0.

M—CHEMICAL EXAMINATION OF THE FAECES

Schmidt's test for activity of fermentation is used after his preliminary test diet (p. 611). The apparatus is shown in Fig. 12. Rub about 5 Gm. faeces into an emulsion with water, place in the wide-mouthed bottle, and fill same with H_2O. Fill the cylinder with H_2O and insert cork bearing the straight glass tube and the side attachment. Fit cork of bottle over the tube, invert all this upper portion of apparatus, and then push cork down into its bottle in such manner that no pockets of air are caught in apparatus. Any overflow of fluid is caught by the side tube, which is open at upper end. Incubate at body temperature for twenty-four hours.

Diastatic Activity.—Winsløw's technique can be applied as for urine (p. 725). A filtered 10% extract of faeces in 0.1% Na_2CO_3 is employed, and results expressed as cc. starch solution digested by 1 Gm. faeces. On a general hospital diet and after $MgSO_4$ or castor oil, McClure and Pratt give the normal value as 0–5000.

Occult Blood.—The precautions noted on p. 720 are especially desirable with faeces.

Gall Stones.—Dry and pulverize the concretion, and extract with alcohol-ether. Upon evaporation, any cholesterol will separate, and the characteristic crystals (Fig. 192) can be recognized microscopically. (See below.)

Bile.—Emulsify a small particle of chlorophyll-free faeces in saturated aqueous $HgCl_2$ on a glass slide with a wooden toothpick. Observe after one or more hours against a white background. Hydrobilirubin, the normal pigment, will produce a salmon-pink color; bilirubin, a green. The residue from the gall stone test above may be treated with cold, dilute KOH to extract any bilirubin. This can then be detected by Gmelin's test, p. 731.

N—DISINFECTANTS AND DISINFESTANTS

By disinfection is meant the destruction of injurious bacteria; by sterilization, the destruction of all living things. Germicides are substances which kill disease-producing bacteria. The term "antiseptic" which has been restricted technically to those substances which are inimical to the growth of bacteria has come to have a wider meaning. The layman will generally buy an "antiseptic" with the idea in mind that it will prevent infection by killing germs. In line with a judicial decision that "Language used in the label is to be given the meaning ordinarily conveyed by it to those to whom it is addressed, the Bureau of Chemistry, U. S. Department of Agriculture, has held that the term 'antiseptic,' when used in the labeling of medicinal substances, is objectionable unless the preparation, when used as directed, will actually render pathogenic microorganisms innocuous."

Deodorants may or may not be antiseptic or germicidal. An insecticide may or may not be a germicide and *vice versa*.

In disinfection we must consider:

1. Strength of solution. It must always be kept in mind that the strength of a germicidal solution when added to an equal amount of material to be disinfected is reduced in strength one-half. Thus 1 pint of a 5% compound cresol solution added to 1 pint of faecal material has a disinfecting effect of a 2.5% solution.

2. Time of application. A common mistake is to consider a few minutes as sufficient for contact of germ-containing material with the disinfectant. In the

faeces-cresol mixture above noted the action of the disinfectant should continue at least one hour before emptying the vessel.

3. Nature of medium in which disinfectant acts. Germicidal agents are much less effective against bacteria contained in material rich in organic matter than when in pure water.

4. Temperature. Disinfecting solutions show greater power as the temperature rises, and act less efficiently in the cold. At 39°C., they are active.

By Coefficient of Inhibition we mean time and concentration necessary to prevent development of bacteria.

By Inferior Lethal Coefficient we mean time and concentration necessary to kill nonspore-bearing bacteria.

By Superior Lethal Coefficient we mean time and concentration necessary to kill spore-bearing bacteria.

Phenol Coefficients.—In determining the germicidal strength of a disinfectant against any given organism it is compared with that of phenol. The Bureau of Chemistry, Department of Agriculture, determines the phenol coefficients of disinfectants under the provisions of the Food and Drugs Act and the Insecticide Act. They are expressed as the *B. typhosus* phenol coefficient, *S. aureus* phenol coefficient, etc., depending on the organism used in the determinations. If more powerful than phenol under the conditions of test the coefficient will be greater than 1. The method employed by the Bureau of Chemistry is based on the Rideal-Walker and the Hygienic Laboratory methods but differs from both in some respects.

Disinfectants may be (A) Physical, (B) Chemical.

(A) Of the **physical disinfectants** we have:

1. Sunlight. The red and yellow rays are practically inert. The ultra-violet most active. Direct sunlight kills non-spore-bearing pathogenic organisms in from one to several hours depending upon moisture, temperature and other conditions. Exposure equivalent to 30 hours sunlight is usually required to kill anthrax spores.

2. Burning. Effective when practicable.

3. Boiling. Efficient. Non-spore-bearing bacteria are killed almost instantly by a boiling temperature but spores may resist destruction for many hours at 100°C. One must remember that the boiling point is lower at mountainous elevations.

4. Steam. Extremely efficient when penetration is insured.

(B) **Chemical Disinfectants.**—*Bichloride of mercury* is usually sold in the form of antiseptic tablets. As a disinfectant for the infectious diseases it is usually used in a strength of 1–1000. The solution should be made in a wooden, enameled or earthenware vessel. As bichloride forms inert albuminates it should not be used in disinfection of sputum, faeces or any albuminous excreta. It must be remembered that bichloride is a mordant so that any stains in soiled clothing will remain permanent. For disinfection of clothing the material should be left in 1–1000 bichloride for one hour. Dishes for food should never be disinfected in bichloride on account of the danger from poisoning. Floors and walls may be disinfected with 1–1000 bichloride applied with a mop. Allow the solution to dry on the floor or walls.

Solution of formaldehyde, U.S.P., contains not less than 37% of formaldehyde A 5% dilution in water (50 cc. sol. formaldehyde 950 cc. water) makes a satisfactory

disinfectant for soiled clothing. It is also valuable for albuminous material. The disinfectant must act in a strength of 5% so that if 1 pint of faeces is to be disinfected we should add to it a 10% dilution of the official solution and allow it to act for one hour.

Fumigation with formaldehyde is employed only when the object is to destroy bacteria as the gas is valueless as an insecticide. Such fumigation is now seldom considered necessary in public health practice. Formaldehyde is efficient as a surface disinfectant when the temperature is above 50°F. and the air contains at least 60% of moisture. Owing to its lack of penetration the gas is not efficient for the disinfection of mattresses, or similar articles.

A convenient method of formaldehyde fumigation is to pour 500 cc. of Solution of Formaldehyde on 250 Gm. of barium dioxide or potassium permanganate for each 1000 cubic feet, allowing exposure for 6 to 12 hours.

In employing this method, take a pan partly filled with water. Place in this a second metal or glass receptacle containing the barium dioxide or potassium permanganate and pour in the Solution of Formaldehyde. The gas is generated in great amounts in a few seconds. The receptacle should be large enough to contain 10 times the volume of the Solution of Formaldehyde, as there is a tendency for the mixture to foam over the sides of the container.

Another practical method is the formaldehyde-sheet spraying one. The solution (37%) should be sprayed on sheets suspended in the room in such a manner that the solution remains in small drops on the sheet. Spray not less than 10 ounces of Solution of Formaldehyde for each 1000 cubic feet. Used in this way a sheet will hold about 5 ounces without dripping or the drops running together. The room must be sealed very tightly in disinfecting with this process and kept closed not less than twelve hours. The method is limited to rooms or apartments not exceeding 2000 cubic feet. The formalin may also be sprayed upon the walls, floors, and objects in the room.

Phenol.—It is soluble in water to the extent of about 5% and in such strength it is an efficient disinfectant. The solution should be made with hot water.

In standardizing disinfectants phenol is used as the standard. It however is expensive and there is often difficulty in making up satisfactory solutions. More efficient and more convenient is the **Liquor Cresolis Compositus, U.S.P.** This may be prepared by mixing equal parts of cresol and soft soap. This has a value according to tests made in the Hygienic Laboratory of 3, making it in tests without organic matter three times as efficient as phenol. Under similar conditions lysol had a value of 2.12, creolin 3.25 and trikresol of 2.62.

Equal parts of a 5% solution of Liq. Cresol. Co. and the faeces, urine or sputum to be disinfected is satisfactory for disinfection provided the mixture is allowed to stand for one hour. Here we would have the effect of a 2.5% solution. Liq. Cresol. Co. (5%) is an excellent disinfectant for contaminated bed clothing, etc. It is also most suitable for the disinfection of floors and walls.

Sulphate of copper.—This salt has a remarkable effect on certain species of algae so that in strengths of 1 to 1,000,000 it is destructive.

Hydrogen dioxide.—A 2% solution will kill anthrax spores in three hours. It is useful in treatment of anaerobic infections, as with the gas bacillus. When hydrogen dioxide is used in the presence of blood or pus, the catalase of the latter rapidly

decomposes the H_2O_2 so that the disinfecting power rapidly disappears. The quality of hydrogen peroxide cannot be depended upon on account of its rapid deterioration.

Lime.—It must be remembered that air-slaked lime is inert as a disinfectant. For disinfecting faeces freshly prepared milk of lime is excellent. It is made by mixing unslaked lime with four times its volume of water. An equal quantity should be added to the faeces to be disinfected.

Chlorinated lime.—This can be purchased in air-tight containers and when the package is opened it should give off a powerful odor of chlorine. Frequently samples fail to yield 30% of available chlorine (the U.S.P. requirement).

For a working disinfectant solution add 1 pound to 2 gallons of water, allow insoluble matter to settle and decant the clear liquid. This is satisfactory for mopping floors and for disinfecting faeces, sputum and urine, equal parts of the excreta and disinfecting solution being mixed and allowed to stand for one hour.

Chlorine.—The water supply for communities is generally treated with chlorine gas for the destruction of the typhoid-colon group. The available chlorine of chlorinated lime and of sodium hypochlorite is also utilized for the disinfection of drinking water. The amount of chlorine necessary to destroy pathogenic organisms will vary from 0.2 part to many parts per million depending on the source of the water, the season, degree of contamination, the amount of organic matter present and other factors. It is considered that the presence of 0.1 part of residual chlorine per million parts of water fifteen minutes after chlorination is an indication that the pathogenic bacteria have been killed. This small excess of chlorine disappears shortly and has no effect on the taste of the water. For determining the amount of residual chlorine, a reagent consisting of 0.1% of orthotoluidine in a 10% solution of sulphuric acid or hydrochloric acid is employed. 1 cc. of this solution is added to 100 cc. of the water to be tested. The color developed in the sample is compared with a series of quantitative colorimetric standards. A non-fragile colorimetric device calibrated for 0.05 part of free chlorine per million and to be used with orthotoluidine is sold in this country for the convenience of those who have not a series of colorimetric standards available. In the field with troops, overchlorination followed by the use of sodium thiosulphate as an antichlor has been practiced in the absence of facilities for the accurate determination of the amount of residual chlorine. Where water is turbid treatment with some precipitating agent like alum is required preliminary to chlorination. Automatic chlorinators for use with either chlorine or chlorinated solutions are now available. They are especially useful for ships operating in fresh water and in the case of water supplied by lighters as the water may be disinfected automatically as it is being pumped on board. Lelean recommends the addition of 2 grams of good quality bleaching powder (chlorinated lime) to the contents of an ordinary water cart which holds 110 gallons of water. This is actually about 1⅓ parts per million of chlorine so that allowing for possible deterioration we can count on 1 part per million being operative. Other agents used for disinfection of small quantities of water are "halazone," sodium bisulphite and calcium permanganate. Halazone tablets have been giving excellent results in the sterilization of the contents of drinking bottles. It must always be remembered that boiling the water is the method of sterilization to be employed when practicable.

Eusol.—A solution containing 0.27% hypochlorous acid and known as eusol has been highly recommended in the treatment of gas gangrene wounds. To make it,

put 12.5 grams chlorinated lime (bleaching powder) in a Winchester quart flask and cover with a liter of water. After thorough shaking add 12.5 grams of boric acid. After again shaking the mixture should stand for a few hours and then be filtered through cotton wool. The clear solution is *eusol*. It must be kept in tightly closed bottles.

Chloramine-T.—This chlorine antiseptic is more stable than hypochlorite solutions and can be used in greater concentration. It is nontoxic and readily soluble in water. It is usually used in 2% solution in the treatment of wounds. Gauze which has been impregnated with a 5% solution and dried can be used in light packing of wounds. In the eye, 0.1% (1–1000) in normal saline is efficacious and nonirritating.

Dichloramine-T.—This, like chloramine-T, is a crystalline substance, but is practically insoluble in water. It is a very active germicide. In use it is dissolved in chlorinated eucalyptol or better still chlorinated paraffin wax. For treatment of infected wounds it is used in 6.5 to 10% strength, the chlorinated oil solution of the antiseptic being sprayed on the wound or gauze covering the wound.

Acriflavine.—Of the dyestuffs recommended as germicides this is better adapted to the purpose than malachite green or brilliant green. Acriflavine, or flavine as it is also called, acts more efficiently in serum mixtures than in aqueous ones and is less injurious to tissue than most other antiseptics. It is generally used in 1–1000 solution in salt solution and makes a good wet dressing when gauze is soaked in such a solution.

Dakin's solution.—The best known and most widely used of the disinfectants of the chlorine group is a neutral sodium hypochlorite solution called Dakin's solution. This contains not more than 0.5% or less than 0.45% NaOCl in a neutral solution. While *eau de Javel* is also a solution of sodium hypochlorite it is irritating on account of its containing an excess of alkali, the caustic action of which has an unfavorable action on wounds. In eusol the boric acid tends to neutralize this prejudicial alkalinity sufficiently to enable the buffer salts of the body fluids to maintain a proper neutral state.

A method of preparing Dakin's solution in the chemcial laboratory of the Naval Medical School is as follows:

(A) Bleaching powder..... 100 Gm.
Water..... 1000 cc.
Shake to mix thoroughly.

(B) Sodium carbonate..... 45 Gm.
Sodium bicarbonate..... 48 Gm.
Water..... 1000 cc.
Dissolve completely.

Mix A and B and shake vigorously for 5–10 minutes or allow to stand in a closed container a few hours. Then filter. This filtrate is the Dakin's solution which will be neutral to solid phenolphthalein (flash of red with alcoholic solution), but will contain about two to three times the amount of NaOCl required. Determine exact per cent. of NaOCl and dilute to proper strength as follows:

Put 10 cc. of the filtered Dakin's solution in a 100-cc. volumetric flask. Add 20 cc. of 10% KI and 2 cc. of glacial acetic acid. Dilute to mark with H_2O and mix thoroughly. Put this wine-colored solution in a burette. In Erlenmeyer flask put 5 cc. of N/10 sodium thiosulphate (24.8 Gm. $Na_2S_2O_3.5H_2O$ per liter of H_2O) and add 2 cc. of starch paste for an indicator. (The starch paste is best made by mixing 1–2 Gm. of starch with about 10 cc. cold water and pouring this into 90 cc. of boiling water.) From the burette run this solution into the 5 cc. of thiosulphate until a faint blue color results. This is the end-point. Take the reading on the burette and calculate the per cent. NaOCl as follows:

Calculation:

$$\frac{18.615}{\text{cc. of solution from burette}} = \text{per cent. NaOCl}$$

$$\frac{50}{\text{per cent. NaOCl}} = \text{the number of cc. of above solution required to dilute to 100 cc. to make 0.5\% NaOCl.}$$

Example:

29 cc. of solution from the burette were required to reach the end-point.

$\frac{18.615}{29} = 0.64\%$ NaOCl.

$\frac{50}{0.64} = 77.88$ cc. So take 77.88 cc. of the Dakin's solution and dilute to 100 cc. This will give 0.5% NaOCl, the required per cent.

Equations involved in the above are as follows:

$$CaCl(OCl) + Na_2CO_3 = NaOCl + NaCl + CaCO_3$$
$$NaOCl + HC_2H_3O_2 = HOCl + NaC_2H_3O_2$$
$$KI + HC_2H_3O_2 = HI + KC_2H_3O_2$$
$$HOCl + 2HI = I_2 + HCl + H_2O$$
$$I_2 + 2Na_2S_2O_3.5H_2O = Na_2S_4O_6 + 2NaI + 10H_2O$$

Disinfestants

By disinfestants we mean those agents used for the destruction of rodents and insects.

Gaseous Disinfestants

Among the fumigants effective against both rodents and insects we have sulphur dioxide, hydrocyanic acid gas, Zyklon-B which was authorized for use in the Public Health Service in 1926, and cyanogen chloride gas. These are destructive to all forms of animal life. Hydrocyanic acid gas and cyanogen chloride gas do not injuriously affect merchandise, textiles, etc. and require less time for exposure than sulphur dioxide. While sulphur dioxide has the manifest disadvantages of rotting fibres of textiles and bleaching certain dyed fabrics, its safety of application makes its use preferable to cyanide fumigants by those inexperienced in large scale fumigation. Prior to fumigation of vessels the crew should be mustered and all absentees

accounted for in order that none might remain in the compartments to be fumigated. Thereafter measures should be taken to prevent the entry of any unauthorized person to such compartments until the medical officer has pronounced them safe for occupancy. In fumigating for rats a check is made after fumigation to insure that no rats remain alive. If necessary, fumigation is repeated. After fumigation, hatches, ventilators, doors, etc. are opened from the outside. The Public Health Service uses "Aerothrusts" (portable gasoline motors with airplane blade fans to remove the gas from holds and spaces having poor ventilation). They do not enter the superstructure for at least 15 minutes after opening up and do not enter the holds until they have been open an hour. In addition a tame rat in a cage is lowered to the bottom of the hold and left for at least five minutes to test for hydrocyanic acid gas or cyanogen chloride gas. If the rat is unaffected, the medical officer goes personally or observes one of his men go through all the compartments before they are declared safe for occupancy. Men doing this carry an anti-cyanide gas mask in the alert position, are equipped with searchlights, and are watched from the deck.

Sulphur dioxide.—For destruction of rats 5 pounds of roll or sublimed sulphur are burned per 1000 cubic feet of space. Shallow pans should be used for the sulphur which is sprinkled with alcohol and ignited. Precautions should be taken against fire by elevating the pans by means of bricks, etc., in a larger vessel containing water. Exposure for six hours is necessary. Two pounds of sulphur per 1000 cubic feet with two hours exposure are sufficient for destruction of mosquitoes, while for body lice four pounds per 1000 cubic feet with six hours exposure are required. Liquefied sulphur dioxide may be employed instead of burning sulphur, two pounds of the gas being substituted for each pound of sulphur. Machinery may be protected from the action of sulphur dioxide by coating the metal parts with lubricating grease. If clothing is washed immediately after sulphur fumigation the rotting effect will be lessened.

Hydrocyanic acid gas.—For destruction of rats 5 oz. sodium cyanide of high purity, 7½ oz. commercial sulphuric acid (66B) and 10 oz. water are required for each 1000 cubic feet of space. Exposure for two hours is necessary. The water is placed in a crock and the acid is run in cautiously immediately before fumigation in order to take advantage of the heat generated. The sodium cyanide, contained in a cloth bag, is dropped in the diluted acid by an operator who wears a special anti-cyanide gas mask. The ordinary military gas mask does not protect against hydrocyanic acid gas and cyanogen chloride. Sodium cyanide "eggs" of the proper weight are to be preferred to the loose cyanide. Liquid hydrocyanic acid in cylinders is efficient but dangerous to transport. For generation of HCN for destruction of insects Creel and Faget employed materials in the following proportions: potassium cyanide 1 part, commercial sulphuric acid (66B) 2 parts and water 2½ parts by weight. The following amounts of potassium cyanide per 1000 cubic feet of space were recommended: for *mosquitoes* 0.4 ounce, 15 minutes exposure; for *bed bugs* 5 ounces, exposure for one hour; for *body lice* 10 ounces, two hours exposure; for *roaches* 10 ounces, one hour exposure.

Zyklon-B.—A granular, earthy substance, the active ingredients being liquid hydrocyanic acid and a persistent lachrymatory gas. It comes in sealed tins of various sizes designed for definite increments of cubic feet of space. The product is poured by men on deck down long U-shaped metal gutters to canvas sheets sus-

pended in the holds and newspapers distributed between decks. Persons pouring Zyklon-B are equipped with anti-cyanide gas masks and a special hammer device for opening the tins. This method is said to be safer and more convenient than the crock method which is used for generating hydrocyanic gas and cyanogen chloride gas.

Cyanogen chloride gas.—This gas has been used as a substitute for hydrocyanic acid gas. Its lachrymatory and irritant properties even in non-lethal concentration are efficient to warn of its presence. It does not injure foodstuffs, textiles, etc. For fumigation against rats the Public Health Service has employed 4 oz. sodium cyanide, 0.8 to 1.6 oz. sodium chlorate (exact amount varies with conditions not yet determined), 17 oz. commercial hydrochloric acid, and 17 oz. of water per 1000 cubic feet of space. The acid and water are mixed in barrels, crocks or buckets and the sacks containing the dry chemicals are dropped into the mixture by men wearing anti-cyanide gas masks. "Eggs" containing a mixture of sodium cyanide and sodium chlorate may be purchased and will obviate the hazard connected with mixing the loose chemicals.

Miscellaneous Disinfestants

Pulicides.—For destruction of rat fleas one must accomplish the simultaneous destruction of rats by fumigation, bait poison, trapping, etc.

Among the liquid pulicides we have (1) crude petroleum (fuel oil) which is at times called Pesterine, (2) an emulsion of kerosene oil made as follows: Kerosene 20 parts, soft soap 1 part and water 5 parts. The soap is dissolved in the water by the aid of heat and the kerosene gradually stirred into the hot mixture.

For *cockroaches* there is nothing so good as sodium fluoride. By sprinkling the powder about the haunts of the cockroaches they are destroyed in a few days.

Pediculicides.—Owing to the great importance of lice in transmitting typhus fever, trench fever and relapsing fever their destruction is a vital consideration.

While the body louse is the important transmitting agent, the head louse and possibly the crab louse should also be destroyed.

The subject of pediculosis has been much discussed on account of its importance among the troops in the European war.

For the destruction of head lice Pernet recommends:

1. Prevention: hair to be kept close cropped and clean.
2. For the nits: wipe them off with a solution of 1 in 30 phenol.
3. For the lice themselves: Unguentum hydrargyri ammoniati, diluted (gr.x to 1 oz.), or any fatty, sticky body well rubbed into the back of the head. Paraffin lamp-oil (kerosene) also good, but not to be used near an open flame or light.

Blanchard considers camphorated alcohol or warm vinegar containing 1 to 1000 corrosive sublimate as useful for head lice. He also suggests the fumigation of clothes with tobacco as valuable for body lice.

For the destruction of body lice Pernet recommends:

1. All body and bed-linen and clothes should be baked or sterilized by boiling.
2. Unguentum staphisagriae should be applied to neck-bands of vests and shirt in the region of the neck.
3. Alkaline baths to soothe the irritated skin.

Sublimed sulphur sprinkled in th · bed and the clothes is very useful.

Castellani and Jackson have gone most extensively into the matter of louse destruction. Their conclusions are as follows: 1. In regard to solid and liquid insecticides, the substances which have been found to be deleterious to body lice are in the order of their efficiency: Kerosene oil, vaseline, guaiacol, anise preparations, iodoform, lysol, cyllin and similar preparations, phenol solution, naphthalene, camphor.

Pyrethrum has a very feeble action on lice, while boric acid, sulphur, corrosive sublimate, and zinc sulphate, when used in powder form, have apparently no action whatever. As regards bedbugs, kerosene oil is the best insecticide. Next to it comes guaiacol, one of the most active drugs of those tried.

For ridding the body of lice the following steps are essential:

1. The hair of the body and head should be clipped.

2. The subject should be bathed, there being used freely kerosene-emulsion soap, prepared by boiling 1 part of soap in 4 parts of water, and then adding 2 parts of kerosene oil. The resultant jelly, when mixed with 4 parts of water, makes a liquid soap that is convenient to use and which may be applied effectively.

3. Following the bath, the body may be anointed with kerosene, special care being devoted to the hairy parts. Skin irritation may, however, require early removal of the oil.

4. It has been found that lice on clothing removed from the body may remain alive nine days and their eggs as long as forty days. The clothing therefore should be disinfested by one of the following methods.

(*a*) Steam; (*b*) boiling for five minutes; (*c*) 5% compound cresol solution for 30 minutes; (*d*) fumigants such as sulphur dioxide and hydrocyanic acid gas. Chlorpicrin has also been employed.

5. In the absence of facilities for carrying out the steps described, or to prevent infestation subsequently, dusting powders are sometimes used. Of these the N.C.I. powder, containing commercial naphthalene, 96 Gm., creosote, 2 cc., and iodoform, 2 Gm., is the most widely known; but Moore's powder—creosote, 1 cc.; sulphur, 0.5 Gm., and talc, 20 Gm.—is less irritating and is said to be six times as effective. It has also been recommended to wring out the underclothes in 5% compound cresol solution, then drying thoroughly, or to impregnate them with substances such as the halogenated phenols.

Raticides.—For large scale extermination of rats, especially during an outbreak of plague, fumigation with sulphur dioxide, hydrocyanic acid gas or cyanogen chloride gas is the most efficient method. For exterminating rats and in this way secondarily the rat fleas, besides the ordinary poisons such as As, P, etc., Rucker has recommended a poison composed of plaster of Paris 6 parts, pulverized sugar 1 part and flour 2 parts. This mixture should be exposed in dry place in open dishes. To attract the rats the edge of the dish may be smeared with the oil in which sardines have been packed. Phosphorus is the base of many of the commercial rat poisons. Its inflammable nature is a drawback. Furthermore rats soon learn to recognize its odor and refuse to eat food containing this poison. Barium carbonate is highly regarded as a rat poison, having neither taste nor odor. It is mixed with meal and bacon fat—about 1 part of barium carbonate to 4 parts of meal. In trapping rats one should frequently change the type of trap, and always allow the traps to be

placed about the storehouse without being set for a day or so, to accustom the rat to its harmlessness. Again they should be handled with gloves so that they may not carry the odor of man. In the use of spring traps every effort should be made to disguise them by covering with saw dust, straw, chaff or meal. Bacterial vaccines, as the Danysz virus, are not considered satisfactory and are dangerous as possible causes of food poisoning in man.

Larvicides.—When drainage and filling of mosquito-breeding areas are not practiaable and the use of fish not possible, various larvicidal agents may be used. The most common of these are petroleum, both crude and refined, Panama larvicide, (p. 564) crude phenol, cresol and a mixture of soft soap and petroleum. Trioxymethylene (paraformaldehyde) has been mentioned by Roubaud and others as being efficient against anopheline larvae. The Marine Barracks at Quantico, Va., reported satisfactory results from the use of oiled sawdust in 1918. Barber and Hayne published an account of the use of Paris green mixed with inert dust to form a surface deposit in 1921. The observation that Paris green is effective against the surface feeding anophelines but has no effect on culicine larvae has been confirmed by subsequent observers. Airplanes equipped with hoppers lend themselves to thorough and economical distribution of larvicides. The Department of Agriculture tried dusting with Paris green from airplanes manned by Army pilots in 1923. Since that time airplanes have been used in various parts of the country to distribute Paris green and oiled sawdust. Oiled sawdust is probably the best general larvicide for use in this manner. Dry fine sawdust is impregnated with a mixture made from equal volumes of crank case oil and kerosene. It takes four to ten days to effect saturation depending upon the kind of sawdust used. Excess oil is permitted to drain from the impregnated sawdust before it is used.

Destruction of Mosquitoes.—Measures of protection against the immediate danger of infection from the adult mosquito merit attention. Screening, fumigation, repellants and swatting constitute the usual means.

Carter stated that wire screening with 16 meshes to the linear inch will exclude anophelines. Number 18 screen (18 meshes to the inch) was adopted on the Isthmus to exclude *Aedes aegypti* which will pass through number 16 screen. It is manifest that screening will not be effective unless particular attention is given to stopping up of cracks around the doors, window screens and elsewhere. Holes through which pipes pass, drains, fireplaces not in use and even key holes should be sealed against mosquitoes. Canvas strips were found by von Ezdorf to be convenient in making window screens and screen doors tight. In quarters for temporary occupancy fine cloth netting nailed outside the windows and secured with battens will do. In infected areas where screening is not possible mosquito bars should be used on beds but must be properly applied to afford protection. They should be suspended so that they hang some distance over the mattress and inside the head and foot pieces of the bed so that the edges may be tucked in snugly under the mattress when one goes to bed. Howard states that the wearing of veils and gloves after sundown enforced at stations on the Italian railroads some years ago resulted in a great reduction of malaria.

The fumigants usually employed to destroy mosquitoes are dry sulphur dioxide, pyrethrum and Mim's culicide (phenol-camphor). The U. S. Public Health Service has also used hydrocyanic acid gas for this purpose.

Dry sulphur dioxide produced by burning 2 pounds of sulphur for every 1000 cubic feet is very effective if the spaces are made tight by stuffing or sealing all cracks and openings. Two hours exposure is sufficient. The vessels in which the sulphur is to be burned should rest on bricks or in a tub of sand to prevent fire but should not rest in water as is done in ordinary fumigation. It is said that in the absence of moisture sulphur dioxide causes no injury to household goods, fabrics or metals.

Pyrethrum is used in the proportion of 2 pounds to 1000 cubic feet. The powder is ignited and after two hours exposure the mosquitoes must be swept up carefully and burned as pyrethrum, while it stuns the mosquitoes, cannot be depended on to kill. The expense is another deterrant to its use.

Mim's culicide is made by triturating equal parts of camphor and phenol. The resulting liquid is volatilized by gentle heat, 4 ounces being used for each 1000 cubic feet. Goldberger states that, like pyrethrum, the fumes of this culicide stun but do not necessarily kill the mosquitoes. Care should be taken not to overheat this substance as the vapor is likely to catch fire. The lamp used to heat the container should stand in a vessel of water for this reason. In the absence of a convenient support, a piece of stovepipe is cut to form three legs. An alcohol lamp is inserted to heat a flat basin resting on the other end of the pipe.

Creel and Faget found that exposure for 15 minutes to the gas from 0.4 ounce of potassium cyanide per 1000 cubic feet of space was sufficient to kill mosquitoes. The amount for 1000 cubic feet yields "approximately 1 part cyanogen to 6000 parts of air, so dilute, in fact, as to practically eliminate all danger to human life. On repeated occasions we entered the fumigating room immediately upon opening the doors after mosquito fumigation without noticing any ill effects." In generating the hydrocyanic acid gas they used potassium cyanide, c.p., sulphuric acid (66B) and water combined in the proportions by weight of 1 part cyanide, 2 parts acid, and 2½ parts water.

For houses that cannot be screened properly by reason of their construction, Coogle found commercial creosote oil to be practical as a repellant for anopheline mosquitoes. The ceilings and walls of 25 houses in various sections of an anopheline-producing area were sprayed in the proportion of 1 gallon of creosote oil to 420 square feet. Anopheline mosquites were found in all the houses on several visits prior to the treatment. After spraying, no anopheline mosquitoes were found in any of these houses during three inspections at three-weeks intervals. Apparently the occupants did not object to the creosote oil and no ill effects were noted in any of those who slept in the rooms subsequent to the spraying.

Volatile oils, particularly citronella, pennyroyal, lavender and cedar, are commonly used on exposed parts of the body as repellants. Spirit of camphor, kerosene, oil of peppermint, oil of tar, lemon juice and vinegar have also been recommended. Samotz recommends dilution with 4 parts of liquid petrolatum to retard the evaporation of oil of citronella. Repellants to be applied to the body are poor substitutes for screening when we have to do with infective mosquitoes.

Swatting the mosquito should be practised more generally. For mosquitoes hard to reach, as on ceilings, a small tin can containing kerosene is attached to a stick. The can is held to the ceiling under the mosquito who, in attempting to fly, will be caught in the kerosene.

O—ANATOMICAL AND PHYSIOLOGICAL NORMALS

In examinations in the pathological or chemical laboratory the following may be considered approximately as normal findings:

1. Anatomical Normals. Averages.

Adrenals. Length, 2.4–2.8 inches (6–7 cm.). Breadth, 1.2–1.4 inches (3–3.5 cm.). Weight, 0.17–0.21 ounce (5–6 grams) each. Left usually larger.

Aorta. Length, varies, 17–20 inches (42.5–50 cm.). Thickness of wall, 0.06–0.08 inch (1.5–2 mm.). Diameter, 0.75–1.25 inches (1.7–3 cm.). Weight, 1.2–1.6 ounces (35–45 grams).

Appendix. Length, quite variable, 3.5–4 inches (9–10 cm.). Diameter 0.25 inch (6 mm.). Weight, 0.25–0.5 ounce (7–14 grams).

Bladder. Capacity, 16 ounces (500 cc.) when normally distended. Thickness of wall, 0.1 inch (2.5 mm.). Weight, 1–2.1 ounces (30–60 grams).

Brain. Weight, female 44–45 ounces (1250–1275 grams), male 48–51 ounces 1365–1450 grams). Length, 6.5 inches (16.5 cm.). Transverse diameter, 5.5 inches (14 cm.). Vertical diameter, 5 inches (12.7 cm.). Dimensions in female being 0.4 inch (1 cm.) less.

Fallopian tubes. Length, 3–5 inches (7.6–12.6 cm.). The right usually the longer. Diameter of lumen averages 0.1 inch (2.5 mm.).

Gall bladder. Length, 3–4 inches (7.5–10 cm.). Diameter, 1–1.25 inches (2.5–3 cm). Thickness of wall, 0.04–0.07 inch (1–2 mm.). Capacity, 1–1.5 ounces (30–45 cc.).

Heart. Weight, female 8.8–9.8 ounces (250–280 grams), male 9.5–12.7 ounces (270–360 grams). Length, 4.5–5.5 inches (11.5–14 cm.). Breadth, 3–4 inches (7.5–10 cm.). Thickness, 2–3.1 inches (5–8 cm.). Thickness, wall left ventricle, 0.35–0.47 inch (9–12 mm.), right ventricle, 0.1–0.12 inch (2.5–3 mm.). Circumference, mitral orifice, 4.1–4.3 inches (10.4–10.9 cm.). Circumference, tricuspid orifice, 4.7–5 inches (12–12.7 cm.). Circumference, aortic orifice, 3–3.2 inches (7.7–8 cm.). Circumference, pulmonary orifice, 3.4–3.6 inches (8.5–9 cm.).

Intestines. Small intestine, length, 22.5 ft. (6.75 meters); $\frac{2}{5}$ jejunum and $\frac{3}{5}$ ileum. Diameter from 1.85 inches (47 mm.) in duodenum to 1.06 inches (27 mm.) at the end of ileum. Large intestine, length, 70.9–76.8 inches (180–195 cm.). Duodenum, length 10.2–11.2 inches (26–28.5 cm.).

Kidneys. Weight, left, 5.3 ounces (150 grams), right, 5 ounces (140 grams). Thickness of cortex, 0.4 inch (1 cm.). Length, 4.5 inches (11.5 cm.). Breadth, 2.50 inches (6.2 cm.). Thickness, 1.25 inches (3.2 cm.). The left longer and the right thicker.

Liver. Weight 50–60 ounces (1440–1680 grams). Greatest transverse diameter, 7.9–9.5 inches (20–24 cm.). Greatest antero-posterior diameter, 3.9–5.9 inches (10–15 cm.). Vertical diameter, 5–6 inches (12.7–15 cm.).

Lungs. Weight, combined, 36–45 ounces (1020–1290 grams). Weight, male right lung, 24 ounces (680 grams), left lung, 21 ounces (600 grams). Weight, female, right lung, 17 ounces (480 grams), left lung, 14.8 ounces (420 grams). Length, 10–12 inches (26–30 cm.). Antero-posterior diameter at base, 7–8 inches (17.5–20 cm.). Transverse diameter at base 4–5 inches (10–12.7 cm.). The

right lung is shorter, broader and thicker than the left. Dimensions in the female average 1 inch (2.5 cm.) less.

Mammary gland. Weight in adult, 5.25–7 ounces (150–200 grams). Weight during lactation, 14–31.75 ounces (400–900 grams).

Oesophagus. Length, 10–12 inches (25–30 cm.). Diameter of lumen, 1.25 inches (3 cm.). Thickness of wall, 0.3 inch (8 mm.). Weight, 1.4 ounces (40 grams).

Ovaries. Weight (each), 0.12–0.25 ounce (4–8 grams). Length, 1.5 inches (3.8 cm.). Breadth, 0.75 inch (1.9 cm.). Thickness, 0.5 inch (1.2 cm.).

Pancreas. Weight, quite variable, 2.1–4.8 ounces (60–135 grams). Length, varies, averages 6–8 inches (15–20 cm.).

Parathyroids. Length, 0.2–0.25 inch (6–7 mm.). Breadth, 0.15–0.17 inch (3–4 mm.). Thickness, 0.05–0.075 inch (1.5–2 mm.).

Pineal gland. Length, 0.4 inch (1 cm.). Breadth, 0.2 inch (5 mm.). Thickness, 0.2 inch (5 mm.). Weight, 3 grains (0.2 gram).

Pituitary body. Length, 0.3 inch (8 mm.). Breadth, 0.5 inch (1.2 cm.). Weight, 5–10 grains (0.3–0.6 gram).

Prostate. Weight, 0.8 ounce (22 grams). Length, 1.25–1.5 inches (3.1–3.8 cm.). Breadth, 1.5–1.75 inches (3.8–4.5 cm.). Thickness, 1 inch (2.5 cm.).

Salivary glands. Parotid, weight, 0.8–1 ounce (25–30 grams). Sublingual, weight, 0.06–0.09 ounce (2–3 grams). Submaxillary, weight, 0.25–0.3 ounce (8–9 grams).

Seminal vesicles. Length, 2 inches (5 cm.).

Spinal cord. Length, 18 inches (45 cm.). Weight, 0.9–1 ounce (27–30 grams). Transverse diameter, averages, 0.5 inch (1.2 cm.). Antero-posterior diameter, averages, 0.4 inch (9 mm.).

Spleen. Weight, 5.5–6.9 ounces (155–195 grams). Length, 4–5 inches (10–12.5 cm.). Breadth, 3 inches (7.7 cm.). Thickness, 1–1.5 inches (2.5–3.7 cm.).

Stomach. Capacity, 1–2 quarts (1–2 liters). Thickness of wall, 0.25 inch (6 mm.). Weight, 4.5–6.2 ounces (125–175 grams).

Testes. Weight, 0.65–0.8 ounce (20–25 grams) each. Length, 1.5 inches (3.8 cm.). Breadth, 1 inch (2.5 cm.). Thickness, 0.8 inch (2 cm.).

Thoracic duct. Length, 15–18 inches (37.5–45 cm.).

Thymus gland. Weight at birth, 0.5 ounce (13.7 grams) and increases to 0.9 ounce (26.2 grams) at end of second year when it gradually decreases until gland disappears. Dimensions at birth, length, 2.4 inches (6 cm.), breadth, 1.5 inches (3.7 cm.) and thickness 0.25 inch (6 mm.).

Thyroid. Transverse diameter, 2.4–2.8 inches (6–7 cm.). Height, 1.2 inches (3 cm.). Weight, 1–1.4 ounces (30–40 Gm.).

Ureters. Length, 11.2–12 inches (28–30 cm.). Slightly longer on left side and longer in male. Diameter of lumen varies, averages 0.1 inch (2.5 mm.).

Urethra. Male. Length, 6.4–8.25 inches (16–20.6 cm.). Prostatic, 1–1.25 inches (2.5–3.1 cm.); membranous, 0.6–1 inch (1.5–2.5 cm.) and the anterior 4.75–6 inches (12–15 cm.). Female. Length, 1.5 inches (3.8 cm.). Diameter of lumen averages 0.25–0.6 inch (7–10 mm.).

Uterus. (Virginal) length, 2.8 inches (7 cm.). Breadth, 1.6 inches (4 cm.). Thickness, 1 inch (2.5 cm.). Weight, 1.4–1.8 ounces (40–50 Gm.). The dimensions of a multiparous uterus are each increased 1 cm. or more and the weight is

increased 0.7 ounce (20 grams). Length of cavity in virgin, 2 inches (5 cm.), in multiparae, 2.25 inches (5.7 cm.).

Vagina. Length, 3–3.5 inches (7.6–8.9 cm.). The posterior wall being slightly longer than the anterior.

II. Physiological Normals (Adult).

Blood: (Values are in mg. per 100 cc. whole blood unless otherwise noted.)

Specific gravity	1.041 to 1.067 (1.026 to 1.032 for serum)
Reaction (see pH on p. 708).	
Total solids	19 to 23%
Haemoglobin	14% (by weight)
Serum albumin	4.6 to 5.3%
Serum globulin	1.8 to 2.7%
Fibrin	0.2%
Total nitrogen	2.6 to 3.5% (plasma, 0.6 to 1.1%)
Nonprotein nitrogen	25 to 35 (plasma, 20 to 35)
Urea nitrogen	12 to 15 (plasma, 10 to 23)
Amino-acid nitrogen	6 to 8 (plasma, 4 to 7)
Ammonia nitrogen	about 0.1
Uric acid (Folin-Wu method)	2 to 3 (extremes, 1 to 3.5) (plasma, 2.5 to 5)
"Creatinine"	1 to 2 (plasma, 0.8 to 1.5)
Creatine	3 to 5 (plasma, 0 to 3.8)
Sugar (Folin-Wu method)	90 to 120 (same for plasma)
Chlorides (as NaCl)	450–500 (plasma, 570 to 620)
Fat (Bloor's fat method)	about 600
Cholesterol (Bloor's method)	140–170
Lecithin (Bloor's "lecithin")	30 (plasma, 22)
Acetone bodies	0 to 4
Bicarbonate (plasma)	53 to 77 vol. % CO_2
Oxygen capacity	18.5 vol. %
CO_2 tension (arterial)	about 40 mm. Hg.
Calcium	5.3 to 6.8 (serum or plasma, 9.0 to 11.0)
Magnesium	2.3 to 4 (serum or plasma, 1.6 to 3.5)
Sodium	170 to 225 (serum or plasma, 335)
Potassium	153 to 240 (serum or plasma, 18–21)
Phosphorus, total (as H_3PO_4)	about 120 (plasma 35 to 40)
Phosphates, inorganic (as P)	(serum) 3.2 to 4.3
Sulphates (as S)	0.5 to 1.0
Diastase (see p. 718).	

Cerebrospinal fluid (see p. 721): (Values are in mg. per 100 cc. unless otherwise noted.)

Specific gravity	1.007 to 1.009
Pressure	7–9 mm. Hg. or 95–120 mm. H_2O (5–7 mm. Hg. in children)
Serum albumin	about 4
Serum globulin	20–30

Urea nitrogen..... 3–13
Creatinine..... 0.4–2.2
Sugar..... 50–80
Chlorides..... 720–750

Stomach contents (see normal acidity curve, p. 744):

One hour after Ewald test meal:

Reaction (see pH on p. 624)
Quantity..... 40 to 50 cc.
Total acidity..... 40 to 60°
Free hydrochloric acid..... 20 to 60°
Pepsin (see p. 745)

Residuum (fasting):

Quantity..... 20 to 100 cc.
Total acidity..... 10 to 50°
Free acidity..... 0 to 30°
Pepsin..... 3 (Mett)
Trypsin..... 7 (Spencer)
Bile..... present in about 60% of cases
Gastric mucus..... traces

Duodenal contents (see pp. 625, 746).

Urine (24-hour specimen; values in Gm. unless otherwise specified):

Quantity..... 25 cc. per kg. body weight
Specific gravity..... 1.015 to 1.025
Reaction..... acid to litmus (pH about 6.0)
Total solids..... 55 to 70
Total nitrogen..... 10 to 16
Urea..... 10 to 40 (supplies about 85% of total N)
Uric acid..... 0.2 to 2
Creatinine..... 1 to 1.5
Ammonia..... 0.5 to 1.2 (see NH_3 quotient, p. 713)
Hippuric acid..... 0.6 to 1
Albumin (see p. 726)
Chlorides (as NaCl)..... 10 to 15
Sulphur (as H_2SO_4)..... 2 to 2.5
Phosphorus (as P_2O_5)..... 1 to 5 (about 90% of phosphates are acid phosphates)
Calcium (as CaO)..... 0.1 to 0.6
Magnesium (as MgO)..... 0.2 to 0.6
Acetone bodies..... about 10 mg.
Sugar (see p. 728)

Faeces:

Average daily output, moist faeces (Hawk)... 110 Gm.
24-hour amount on ordinary mixed diet..... 110 to 170 Gm. (25 to 45 Gm. solids)
24-hour amount on vegetable diet..... to 350 Gm. (about 75 Gm. solids)

Fatty substances (averages expressed as per 1 Gm. dried faeces):

Total fat	225 mg.
Total fatty acid	86 mg. (37.9% of all fat)
Total soap	74.7 mg. (33.4% of all fat)
Total neutral fat	64.4 mg. (28.5% of all fat)

Respiration:

Alveolar air:

Oxygen	14.5%
Carbon dioxide	about 5.5% (35 to 40 mm. CO_2 tension)
Nitrogen	80%

Air hunger in diabetes or chronic nephritis begins only when CO_2 tension has fallen to 20 or 25 mm.

P—THE PROBLEMS OF NUTRITION IN HEALTH AND DISEASE

A few years ago the problem of proper nutrition seemed to rest in providing each day protein, fat and carbohydrate in such quantities that the whole diet would have a certain fuel value—about 3000 calories for an adult performing physical work, less for a sedentary person, more for a laboring man. In addition it was recognized that our water requirements were of prime importance and that such inorganic elements as chlorine, phosphorus, calcium, sodium, potassium, magnesium, iron and iodine, variously combined, also were necessary, some of them being required for proper nutrition and others to bring about processes of oxidation or to maintain osmotic equilibrium.

Later, it having become evident that not all members of a class of food stuffs are of equal value to the economy, the subject of the superiority of certain proteins over others received much attention, and, from the studies made, it appears that the value of these tissue-building substances varies with their amino acid content. As a method of indicating the degree to which the several proteins are utilized, i.e., their biological protein value (B.P.V.), the value of meat protein is taken as unity, and other proteins are assigned a figure denoting the relative quantity required to replace physiologically meat protein. Thus, the relative amount of zein, the principal protein of maize, required to replace meat protein, is 3.4.

Investigations, initiated and stimulated by the experiments of Eijkman (1897 to 1905) on the neuritis-preventing properties of the portion of the rice grain removed in milling (rice polishings), have demonstrated the existence of certain accessory food substances, commonly designated vitamins, and have proved that they are necessary constituents of an adequate diet. At the present time we recognize the following vitamins: (1) Fat soluble A, (2) water soluble B, (3) water soluble C, (4) vitamin D which is formed in the animal organism under the influence of ultraviolet light and is found in cod liver oil, (5) a vitamin variously referred to as E or X by different investigators, a dietary factor influencing reproductive power, and (6) a factor designated by Goldberger as the pellagra preventive (P.P.) factor. Vitamin D, formerly confused with fat soluble A as contained in cod liver oil, plays

an important role in the prevention of bone pathology, especially that characterizing rickets. Adequate exposure to sunshine or the ingestion of this vitamin in cod liver oil seems to have an important function in promoting storage of calcium. It appears that the ratio of calcium to phosphorus ingested is also of importance in conjunction with the action of vitamin D, but inasmuch as the optimum amounts of calcium and phosphorus in the diet of the adult are still unknown, it is not possible to state exactly what the ratio should be. It seems likely that the intake of calcium and phosphorus, and possibly their ratio, affect ossification in general and play an important part in the etiology of dental caries and other tooth defects. There is also evidence that the internal secretions are as intimately concerned in the normal utilization of foods as in the causation of those diseases of metabolism commonly recognized as being due to endocrine disturbance.

The Fuel Needs of the Body

In specifying the potential energy contained in different classes of food substances, we use as the unit of measurement the amount of heat required to raise the temperature of 1 kilogram of water 1°C. This unit of heat is one large Calorie. The fuel value of a food is determined by burning 1 gram in a bomb calorimeter. Most foods yield more heat when thus rapidly combusted than in the course of metabolism. The average number of heat units yielded by proteins as ingested in ordinary diets is now generally considered to be 4 Calories per gram. Under the same conditions a gram of fat is considered to yield 9 Calories and each gram of carbohydrate 4 Calories. The factors of Rubner, the pioneer investigator, indicating utilizable fuel values, were, protein, 4.1; fat, 9.3; and carbohydrate, 4.1 Calories per gram.

Infants and children have proportionately higher caloric requirements than adults. Thus under one year of age there should be given food having a caloric value of 100 per kilogram (2⅕ lbs.) of weight; for a child of six years, from 70 to 80 Calories; for one of twelve years or above, 50 Calories, and for an adult from 30 to 44 Calories per kilogram of body weight. An adult weighing 70 kilograms (156 lbs.) would require, according to the above standards, foods with a fuel value of from 2100 to 3080 Calories per day.

Basal Metabolism.—A fairly constant relationship has been found to exist between the quantity of heat produced and the surface area of the body. The surface area may be calculated by means of the Du Bois formula from the individual's height and weight. A healthy adult who has not had food for fifteen hours, and who remains quietly in bed, produces about 40 Calories per square meter of body surface per hour. Thus, a healthy person, weighing 70 kilograms, under the conditions of basal metabolism shows a heat production of about 70 Calories per hour or 1680 in twenty-four hours.

Lusk gives the following figures as daily fuel requirements:

In bed 24 hours	1680 Calories
In bed 8 hours; in chair 16 hours	2170 Calories
In bed 8 hours; in chair 14 hours; exercise 2 hours	2500 Calories

For those at hard work the caloric needs vary from 3500 to 4500 Calories per day.

As noted previously the requirements for children are higher than those of adults as given above, and those for elderly persons may be estimated as 10 to 20% lower.

In a well balanced diet the daily fuel requirements for a 70-kg. adult, doing moderate work, are provided by 400 grams of carbohydrates and 100 grams each of fat and protein.

400 grams carbohydrate	1640 (400 × 4.1)
100 grams protein	410 (100 × 4.1)
100 grams fat	930 (100 × 9.3)

Such a diet would furnish 2980 Calories.

Analysis of a Daily Dietary.—Estimating the food value of the usual three meals, with the breakfast heavy as is common among Americans, we might consider the following as an example of a diet well supplied with proteins of high biological value, ample provision for mineral salts and with abundant supply of the necessary vitamins. While somewhat high in protein and fat it is fairly satisfactory for a man doing light work. The liver, leafy vegetables, butter and milk ensure ample fat soluble A, while the fruits, lettuce, tomatoes and milk furnish an abundance of water soluble C. The water soluble B is more than adequately cared for in the spinach, tomatoes, liver and milk. The pint of milk alone would about supply one-half the calcium requirements, and the spinach is rich in iron.

Breakfast: Half grape fruit with sugar.
Oatmeal with cream and sugar.
One egg.
Bread (2 slices).
Butter—small ball.
Cream and sugar in cup of coffee.

This breakfast has a fuel value of 1000 Calories, divided as follows: Protein 91, fat 320, carbohydrate 589.

Luncheon: Orange.
Liver and bacon.
Mashed potatoes.
Bread (2 slices).
Apple pie (very small piece).
Pint of milk.

The luncheon has a fuel value of 1010 Calories, divided as follows: Protein 197 fat 342, carbohydrate 471.

Dinner: Celery soup (plate).
Bread (2 slices).
Roast beef.
Boiled potatoes (2 small).
Spinach.
Stewed tomatoes.
Lettuce with French dressing.
Rice pudding.
Water.

The dinner has a fuel value of 1015 Calories made up from protein 130, fat 422 and carbohydrate 463. From protein we have 418, from fat 1084 and from carbohydrate 1523 Calories. The fuel value for the three meals is 3025 C., obtained from 102 Gm. protein, 116 Gm. fat and 371 Gm. carbohydrate. This is more than is required by many adults.

The amount of water to be taken daily either with meals or at other times, and including water in various foods, should approximate 5 pints.

In the selection of food, unless we have an excess, we may encounter a deficiency in protein, vitamin or mineral substance, so that until the art required in the selection of a well balanced diet is better appreciated it is advisable to err on the side of excess over our true needs. The economic feature of the daily ration is one of the greatest importance to most of us and it is of interest to note that a diet composed of milk, cod heads stuffed with liver, turnips, potatoes and oatmeal, which articles constitute almost the sole food supply of the poor crofters of the Hebrides, suffices to maintain the excellent health observed in these people.

The Proteins of the Diet

The protein requirement is absolute, not relative. With a high total fuel value a given amount of protein will, of course, represent a smaller percentage of the total than if less food is eaten. For example, 100 grams of protein furnishes about 16⅔ per cent. of the total fuel value of a diet yielding 2400 Calories, while in a diet of 3000 Calories the same amount represents 13⅓ per cent. Seventy-five grams of protein in a 2400 Calorie diet represents 12½ per cent. and with 3000 Calories, 10 per cent. Milk has been regarded by some as furnishing a clue to the proper proportion. In human milk 8 to 12 per cent. of the total are protein calories, but in cow's milk proteins furnish 18 to 20 per cent. Milk protein has a high biological or growth promoting value. If all the proteins of a diet were of equally high value a smaller intake of protein than contemplated above would probably suffice, but in view of the fact that many vegetable proteins are of much lower value it would seem that we might consider an amount between 75 and 100 grams as sufficient to meet the needs of most individuals.

In estimating the protein value of a food we determine the amount of nitrogen and multiply the figures obtained by 6.25, as nitrogen makes up approximately 16% of a protein. As a matter of fact, however, there is a variation in the nitrogen of different proteins, this being higher for vegetable proteins. There are also other factors of error, as some of the nitrogen in a food is present as extractive nitrogen and not as part of the protein.

Protein Needed for Tissue Building and Repair.—It is true that proteins on oxidation furnish energy, as do the fats and carbohydrates. The body can store protein or amino acids only to a limited extent and, depending upon constitutional factors, any excess of protein ingested will be burned and the nitrogen excreted. As pointed out by Rubner, proteins have greater power to increase heat production in the body than corresponding quantities of fats and carbohydrates. Rubner discovered this phenomenom which he termed the *specific dynamic action of protein*. Protein foods fortify us against cold so that inhabitants of Arctic regions instinctively prefer such foods. Those living in the tropics do not require more protein than will suffice for tissue growth and repair.

The chief consideration as to proteins rests in their utilization by the tissues and in this respect we find a great variation in their value. Before a protein can be utilized it must be broken down into its constituent amino acids. There are about eighteen of these amino acids which have been obtained upon analysis of various proteins, some of which, as lysine and tryptophane, are known to be indispensabls to life. We know that the protein gelatin will not suffice to restore tissue lose because, as shown by its analysis, it is wanting in tryptophane, cystine and tyrosine. Amino acids are absorbed from the alimentary tract and synthesized by the body cells to form tissue but it would seem that only certain amino acids are indispensable. According to Hawk, cystine, lysine, tyrosine and tryptophane cannot be synthesized in the animal body and must be supplied in the diet.

Proteins vary greatly in their amino acid content, some having certain of these "building stones," as they are called, in insufficient amounts or not at all. If a protein contains too little of an essential amino acid the deficiency may be made up by ingesting a greater amount of this protein or, if entirely lacking, by adding to the diet another protein which does contain the lacking amino acid or acids. Zein, one of the proteins of maize, is deficient in tryptophane and lysine, and the protein of wheat, gliadin, has an excess of glutamic acid but does not contain lysine.

The value of a protein seems largely to depend, as stated, on the kind and quantity of amino acids it contains. Meat and milk proteins have a very high value because they contain all the amino acids which enter into synthesis of the protein of body cells. These are sometimes called superior proteins to distinguish them from the inferior proteins of corn, wheat and beans. Thomas gives the following as minimal amounts of well known proteins sufficing to protect the body protein from loss: Meat, 30 grams; milk, 31 grams; rice, 34 grams; potato, 38 grams; bean, 54 grams; bread, 76 grams; and corn, 102 grams. Wilson, however, believes that while 30 grams of meat protein is sufficient to maintain nitrogenous equilibrium, 40 grams is to be considered a minimum B.P.V. (biological protein value).

Nitrogen Equilibrium.—If the amount of nitrogen taken in as food just balances the amount excreted, the body is in a state of nitrogenous equilibrium. There may be nitrogenous equilibrium at different planes of nutrition. That is, although the amount of nitrogen excreted may just balance the intake when only sufficient protein is ingested to meet tissue needs, there will also be equilibrium if a much greater amount is eaten. We find the normal adult in a state of nitrogenous equilibrium while the growing child excretes less than the amount ingested.

By the study of the nitrogen balance of a group of students, soldiers and instructors, Chittenden found that with superior proteins the amount which would maintain nitrogen equilibrium could be reduced even to 30 grams. Of course with inferior proteins the amount would have to be greater. That such a low protein diet is sufficient seemed to have been proved during the war in certain European countries, as by Hindhede, in Denmark. At the same time there are so many factors, such as lowered resistance to infection, decreased appetite, etc., which are difficult to estimate, that it would seem advisable to accept as minimum standard of protein requirement an intake of from 80 to 100 grams of protein. This amount would amply cover deficiencies of certain vegetable proteins. It is always well to supplement proteins from cereals or legumes with milk, egg or other animal proteins. An excess of protein does not seem to carry the dangers that have been attributed to a

high protein diet, and McCollum states that such evil effects as appear to follow are to be attributed in many instances to faults in the diet not connected with its protein content.

Liver and kidney have a high B. P. V. and in addition are of great value as vitamin carriers. It is true they yield a large amount of purins, and, ultimately, uric acid, for which reason they should be avoided by the gouty.

The Purin Bases.—Among the nitrogenous substances of certain diets we have the purin bases, guanin, adenin, xanthin, hypoxanthin and uric acid. These bases occur combined with nucleic acid in the nuclei of animal and plant cells. They are particularly abundant in the nuclear protein of glandular organs and legumes, and hence such foods should be avoided by the gouty. On a purin-free diet a man excretes from 0.3 to 0.4 gram of uric acid, which, coming from the breaking down of his own cells, is called endogenous purin. The purin derived from the food is called exogenous purin and varies from 0.2 to 0.5 gram, according to the character of the diet. These purins do not seem to be necessary for the formation of all nuclei as these are formed normally on a purin-free diet. Caffein and theobromin are methyl purins but the ingestion of coffee or tea, which contain them in large percentage, does not seem to increase uric acid and therefore these beverages need not be forbidden in the diet of the gouty as are other purins. In foods the highest percentage of purins is found in sweetbreads, 0.4. Liver has a little more than 0.1%. The percentage for meats is about one-half that for liver. Beans have about one-half the content of meats, and most other vegetables, excluding the legumes, have only traces or none at all.

Carbohydrates and Fats

In discussing the fuel requirements of the body it was noted that proteins as well as carbohydrates and fats may provide the caloric needs of the body; but, since the proteins alone have the power of tissue formation and repair, they should, perhaps, be reserved for those functions and *protected* from demands to supply energy by the ingestion of adequate amounts of fats and carbohydrates. Muscular energy with its attendant production of heat should be mainly furnished by the carbohydrates and, to a less extent, by the fats. Fats are almost entirely absorbed in the alimentary canal. Carbohydrates usually are completely absorbed three or four hours after ingestion, while the fact that the chief absorption of fats takes place five or six hours after being taken, accounts for their "staying power." According to Starling, individuals deprived of fats are liable to get hungry before the next meal and their work and efficiency suffers accordingly.

In estimating the value of any diet it must be understood that its utilization by the body is never complete but varies in degree with the type and source of the food stuffs even when the processes of digestion are normally performed. Approximately 97% of the proteins of animal origin are absorbed while with vegetable proteins the average amount absorbed is about 84%. With fats likewise the animal fats are more absorbable than the vegetable ones—95% for the former and 90% for the latter. There is less difference with carbohydrates from various sources. According to Atwater, in a mixed meal about 92% of protein, 95% of fat and 97% of carbohydrate are assimilated; hence, by multiplying the several weights of the mixed

protein, fat and carbohydrate ingested by 0.92, 0.95 and 0.97, respectively, we obtain the amounts *utilized*.

Nutritive ratio.—The proportion of the utilizable proteins to the combined carbohydrates and fats should be about as 1 to 6, and that may be considered a normal nutritive ratio. In a well balanced diet for a man weighing 70 kilograms doing light work it is usually stated that, of 600 grams total, 400 should be furnished by carbohydrates and 100 each by protein and fat.

Carbohydrates.—The designation carbohydrate has rather a physiological than chemical significance and in food metabolism the term is usually limited (1) to the simple sugars, such as glucose or grape sugar and levulose or fruit sugar, and (2) to the complex sugars such as the disaccharids (saccharose, lactose and maltose) and the polysaccharids (starch, dextrin and glycogen). The amino sugars (as glucosamin) probably represent a transition from the amino acids of protein to carbohydrate. The simple sugars can be absorbed as such and eventually stored up in the liver as glycogen, but the disaccharids and polysaccharids must be broken down by digestion into glucose and levulose before they can be absorbed.

Of course the main source of carbohydrates for metabolism is from the carbohydrates taken in as food but it must not be forgotten that carbohydrates may be derived from proteins.

In estimating the carbohydrates of a given diet it must always be kept in mind that vegetables lose a large part of their carbohydrate in the water in which they are boiled, and if this water is thrown away most of the carbohydrate is lost. Thus Van Noorden has shown us that, of the 2.97 grams of carbohydrate in 100 grams of fresh spinach, 2.12 grams is dissolved out and lost in the water in which it is boiled. This explains why the water in which vegetables have been boiled has a food value. As this water is rich in carbohydrates, salts and flavoring substances, it should be conserved by using it as stock for soups and sauces.

Fats.—The fats are glyceryl esters of fatty acids. In digestion the fats are acted on by ferments called lipases and undergo cleavage into glycerine and fatty acids. These fatty acids react with alkali to form neutral soaps and as such are absorbed and stored in the tissues. The unabsorbed fat passed out in the faeces varies with different sources of food but on the average amounts to only about 5% of that ingested. In the important tropical disease sprue as much as 25% of the ingested fat may be lost in the stools.

Fats not only serve as sources of reserve fuel by being stored as fatty deposits in the body but also are utilized in the formation of the lipoidal constituents of body tissues, chief of which are phosphatids and cholesterols. Faulty metabolism of fats gives rise to ketosis, or the presence of ketone bodies in the blood, a condition apparently associated with failure of the antiketogenic activity of carbohydrate substances. Fats must be adequately balanced by antiketogenic constituents of the diet (carbohydrates) to prevent ketosis and ketonuria.

The Mineral Constituents of the Diet

About 99% of the elementary composition of the human body is made up of oxygen (65%), carbon (18%), hydrogen (10%), nitrogen (3%), calcium (2%), and phosphorus (1%). The remaining 1% is composed of potassium (0.35%), sulphur

(0.25%), sodium (0.15%), chlorine (0.15%), magnesium (0.05%), and iron (0.004%). Very minute amounts of iodine, fluorine, and silicon are also present, and possibly traces of manganese and aluminum.

With the exception of oxygen, these elements are derived from food. Certain of them (sodium, potassium, calcium, and magnesium) constitute the base that is so necessary for the neutralization of the acids formed in metabolism, such as carbonic, phosphoric, sulphuric and hydrochloric, these being derived essentially from carbon; phosphorus, sulphur and chlorine, respectively. Whether a food will be predominantly an acid-former or a base-former in metabolism depends upon which group of these elements is found most abundantly in its ash. Cereals, flesh, and eggs are acid-forming, while fruits, even those we call "acid fruits," and vegetables are base-forming. The food can similarly affect the reaction of the urine, although it is well to remember that plums, prunes, and cranberries tend to produce an acid urine, despite their basic ash, by reason of their content of benzoic acid which is synthesized to hippuric acid by the kidneys.

These mineral constituents are metabolized, and, under average conditions of health and diet, a man excretes daily about 20 to 40 grams of mineral salts, chiefly chlorides, sulphates, and phosphates of sodium, potassium, calcium, and magnesium. These, except for sodium chloride, represent a real loss of base as the result of acid neutralization, and it is vital that the diet replace these lost metals.

Sodium and Potassium Metabolism.—Sodium occurs in the circulating fluids mainly as the chloride and bicarbonate, while potassium is present chiefly as the phosphate. They are of importance in the regulation of the reaction of the body fluids and tissues (see p. 709), and in the control of osmotic pressures, and thus the distribution of fluids.

While the accepted estimate of the daily NaCl requirement is but 3 or 4 grams, yet the average consumption approximates 15 to 20 grams daily, indicating that the salt is taken voluntarily as a condiment rather than as a food. Normally the surplus is eliminated, but one should remember that an excessive intake tends to cause retention of water (oedema). Reduction of intake decreases the salt content of the tissues, and causes elimination of water, but even on a salt-free diet a point is soon reached where the body jealously guards its salt store and practically none leaves by way of the urine.

The vegetarian or the herbivorous animal requires more sodium chloride than the meat-eating man or animal because the high content of potassium in vegetable foods causes an increased elimination of sodium chloride.

Sulphur Metabolism.—Almost all the sulphur utilizable for metabolic purposes is derived from our protein foods, particularly those containing cystine; inorganic compounds cannot be used as a source of body sulphur. With a protein intake of 100 grams, we would obtain about 1 gram of sulphur, an amount which would suffice for the needs of the body. The sulphur is oxidized during metabolism, producing sulphuric acid which must be neutralized by the bases. Evidence is accumulating that sulphur in reactive form as in cystine or the dipeptide, glutathione, not only plays an important role in tissue utilization of oxygen but also serves as a detoxicating agent. The high reactivity of the sulphur compounds of the body is bound to a large degree with the sulphydryl group. It is probably this system that makes cysteine, the reduced form of cystine, effective as a detoxicating agent.

Metabolism of Calcium and Phosphorus.—Almost all investigators stress the probability of calcium deficiency in the present-day diet. The accepted minimum requirement is 0.45 gram of calcium, and it has been estimated that the diet should furnish at least 1 gram of calcium oxide (the equivalent of 0.67 gram of calcium) daily. Sherman gives 0.73 gram as the average calcium content of American dietaries, but it should be remembered that the calcium in different foods is apparently not equally assimilable. That in milk seems to be particularly efficient. McCollum states that the optimal amount of calcium in the diet of the adult is still unknown. It would seem that the amount, and especially the proportionate amount, of calcium advantageously ingested will depend upon the phosphorus and vitamin D content of the diet. He considers that "any diet will be better for containing more calcium in actual per cent. of the element than it does of phosphorus, and this the average American diet does not do unless the calcium-rich foods are abundantly supplied." Practically it is impossible to have more calcium than phosphorus in the diet without adding calcium to the food, and that, as a rule, is neither necessary nor wise for healthy persons. The necessity for adding calcium salts to the food presupposes insufficient ingestion of foods which are naturally good sources of this mineral. It should be remembered that these are the so-called protective foods which are the main sources of other essentials as well as calcium. The utilization of calcium and phosphorus depends to such an extent upon sunlight or the action of vitamin D that calcium and phosphorus metabolism is discussed in more detail in connection with that vitamin on page 778.

Children require much greater amounts than adults, and there is an extra need during pregnancy. There does not seem to be any objection to an excessive intake of calcium, so that it may be better to exceed than to attempt merely to meet the minimum requirements.

Much of the calcium in food is lost during cooking, and its absorption may be decreased by alimentary tract disturbances. When milk is boiled, a large part of the calcium is lost in the scum, which takes up and retains the calcium phosphate. Probably little or no calcium is lost when milk is properly pasteurized. Milk is our best food for the supply of calcium, an intake of 1000 grams supplying about 1.2 grams.

Meat is very poor in calcium, and commercial wheat, rice and corn products, such as highly-milled rice, patent flour, etc., lose in the processes of manufacture approximately half of the original calcium content. Sherman notes that the amount of calcium per 3000 calories is only 0.18 gram in meat, while it is 5.2 grams for milk. On the same scale, eggs have a content of 1.35, wheat grain 0.4, wheat flour 0.18, oatmeal 0.5, dried beans 1.4, potatoes 0.5, cabbage 4.3, oranges 2.6, and polished rice 0.04 gram.

Administration of phosphates will cause an excretion of calcium, and the reverse also holds true.

It now seems well established that inorganic phosphorus, as phosphates, may suffice to meet the needs of the animal for phosphorus. Still, it is possible that a minimal amount in organic combination is required, although rat experiments would seem to negative this view. Sherman gives the phosphorus requirement as 1.44 grams (3.3 Gm. P_2O_5) for the daily ration, a phosphorus "standard" corresponding to a protein "standard" of 75 grams. Eggs, especially egg yolk, are the best

sources of phosphorus. One egg yolk would furnish about 0.2 gram P_2O_5. A glass of milk would contain about 0.3 gram P_2O_5.

Iron Requirements.—Iron is an essential constituent of haemoglobin as well as of the chromatin substances, and plays an important part in oxidation and catalysis of enzymes. The iron of the food is absorbed from the small intestines, and enters the lymph stream to be deposited mainly in the liver, spleen and bone marrow. Apparently, inorganic iron is assimilable as well as that in organic combination, but this matter is not fully decided and it would seem safer to depend on the organic iron of food stuffs. Some think the inorganic iron to act as a stimulator rather than as a source of tissue iron. In 1927, Simmonds, Becker, and McCollum reported the results of experiments which seem to show that vitamin E is specifically concerned with the assimilation of iron.

It is usually stated that we need from 10 to 12 mg. of iron daily, but growing children and women (by reason of menstruation, pregnancy and lactation) require more, probably up to 15 mg.

While 100 grams of lean meat contains almost 4 mg. of iron, yet meat iron does not seem as valuable as the form found in eggs, milk, or vegetables. A striking fact about milk is its low iron content, there being only about 0.24 mg. in 100 grams. Using the same comparison, lean meat gives 3.85 mg., egg yolk 8.6, whole wheat 5, wheat flour 1, oatmeal 3.8, spinach 3.6, potatoes 1.3, and cheese 1.3. It should be noted that most of the iron of cereals is lost in milling. The iron content of spinach is notably high, and fruits and vegetables are our most important sources of iron. Egg yolk is also a valuable source of iron.

Infants fed on modified cow's milk run a great danger of becoming anaemic by reason of deficiency of iron.

Iodine Requirements.—Iodine is chiefly found in the thyroid gland, and is a constituent of an organic compound found in the gland and known as thyroxin, which is important in the regulation of metabolism. It would seem probable that the frequency of goitre in certain areas (Great Lakes region) is connected with a lack of iodine in the waters and soils of such regions. Striking results have been obtained by giving sodium iodide to school girls where goitre is particularly prevalent.

Irish moss and agar-agar contain iodine.

Water and Metabolism

When we consider that water makes up at least 60% of the body weight, this fact alone should suggest its importance for purposes other than to serve as a diluent for solid food. We know that a man can live without food for at least a month, but, without water, one can live only a few days. Water is concerned with the osmotic equilibrium of the tissues, serves as a solvent for the various substances so necessary to proper metabolism, acts as the vehicle for carrying off waste products, and, by its evaporation from the skin, aids in the regulation of body temperature.

In considering the water balance of metabolism, we have, as sources of supply (1) the drinking water, (2) the water of our food and (3) the water resulting from the oxidation of fats, proteins and carbohydrates. This latter source supplies about 12 cc. per 100 calories, or, on a 3000 caloric diet, 360 cc. In the output, we have (1) that excreted in the urine, (2) the amount lost in the faeces, which has been estimated

to average about 100 cc. daily, and (3) that lost in the sensible or insensible perspiration and in the expired air. Atwater and Benedict estimate that man at rest in a comfortable room loses about 600 cc. by insensible perspiration, and 400 cc. in the expired air. Of course a small amount of water is lost each day through the medium of the nasal secretions, tears, sputum, etc.

From the above it will be seen that the daily water requirements average about 3 liters or quarts—more, of course, when active exercise is taken or in hot weather. Apart from the water obtained from our food stuffs and that from their oxidation, we should drink ordinarily from 5 to 6 glasses each day (3 pints). A cup of coffee or a glass of milk can replace an equivalent amount of water. It is a question whether the excessive drinking of water practised by some persons (12 to 15 glasses daily) is not injurious, and such excess should certainly be avoided by those with damaged heart or kidneys. The idea that digestion is carried on better with a reduced water intake is not sound, even when applied to the taking of water with meals, as it is probable that water drinking promotes the secretion and efficiency of gastric juice, bile and pancreatic fluid.

The Vitamins

The conception of a food deficiency disease, other than a state of defective nutrition due to insufficient intake of protein, carbohydrate, fat or mineral salts, may be said not to have existed prior to 1910. It is true that Eijkman, in his experiments commenced in 1897, showed that polyneuritis of fowls could be produced by feeding them on a diet of polished rice, and that the disease could be prevented or cured by adding to the diet the material removed from the rice during the process of milling (rice polishings); but his paper, published in an obscure journal, remained unknown until attention was called to it by the chemist Funk who, in 1910, demonstrated the need of a certain constituent of the diet to which he gave the name "vitamin," and also laid emphasis on the infinitesimal amounts of the substance required for the maintenance of health. At first, he believed that he had isolated it, but in this he was in error. It may be stated that up to the present time vitamins have not been obtained in pure form. Even when enormously concentrated, they still lack the characteristics of chemically pure substances.

For many years it was thought that animals could live and thrive on purified food stuffs, and it was even suggested that a diet of such components might have advantages. In 1909, McCollum reported success in feeding young rats on a diet of purified proteins, carbohydrates and fats with suitable inorganic salts. Later on Osborne and Mendel, altering McCollum's diet only to the extent of substituting lard for butter and purified starch for lactose, repeated the experiments without success. McCollum, in investigating the discrepancy in results, found that he had used lactose which was not chemically pure and retained some of a neuritis-preventing substance present in the milk. With a pure lactose his results too were not satisfactory. He found further that butter fat contained some essential substance not present in lard. Believing at that time that these two "accessory food substances" were the only ones required in a diet, he named the fat soluble one A and the water soluble one B. Since rats, the animals with which he worked, seem not to require the antiscorbutic vitamin (water soluble C), McCollum failed to note the necessity

for this vitamin in the diet of most animals. It may be stated that Hopkins, in 1912, reported that rats would show normal growth on a "standard" diet if very small amounts of milk were added.

Apparently all vitamins are necessary for growth. There is no particular reason for speaking of any one of them as the "growth vitamin" although of late attention has been directed especially to the growth promoting action of vitamin D.

In making experiments in connection with vitamin A, the albino rat is usually considered the most suitable animal; for vitamin B, the pigeon or chicken; for C, the guinea pig, and for the anti-rachitic vitamin, the rat or young puppy.

Fat Soluble A. (Vitamin A).—This vitamin is often called the anti-ophthalmic vitamin because its absence from a diet leads to an eye disease, xerophthalmia, in a large proportion of experimental rats. The primary lesion appears to consist in more or less complete inhibition of the lachrymal glands—a peculiar phenomenon observed also in the salivary glands. It is now well recognized that a deficiency of A is responsible for the terrible incidence of this eye condition in the children of India and for night blindness also. It is thought also that vitamin A deficiency increases susceptibility to various lung, skin and other affections. This vitamin is abundant in milk and eggs as well as in the glandular organs, such as liver or kidney. In butter it appears in much greater concentration. Lard or the vegetable oils, practically, do not contain it. In muscle it is present not at all or only in minimal quantities. Alfalfa is rich in vitamin A and the milk of cows fed on this grass shows a high content; in fact, the richness of cow's milk in this vitamin depends on the source of the animal's food. Tomatoes, spinach, chard and, to a less extent, lettuce are rich in vitamin A. McCollum has designated milk and the leaves of plants as "protective foods."

Chlorophyll seems to be a most important factor in the elaboration of vitamin A. Green sea-weeds contain it, and this may be the original source of the extraordinary content of vitamin A in cod-liver oil, which possibly has one hundred times as much as does a similar amount of butter. Man and animals seem to have the capacity to store this vitamin and to draw on their reserve in periods of its deficiency in a diet.

A concentrated preparation of vitamin A may be obtained from the unsaponifiable matter removed from cod liver oil, if precaution is taken to avoid the destruction of the active principle which will result from oxidation unless the chemical treatment is carried out in an atmosphere of nitrogen. Drummond states that a liter of cod liver oil contains 8 or 9 grams of unsaponifiable matter, about 50 per cent. of which is cholesterol. The cholesterol may be removed without reducing the vitamin A activity of the remaining fraction. The unsaponifiable matter from cod liver oil carries the whole of the vitamin A and of the vitamin D content of the raw oil. The concentrate contains no detectable trace of iodine or of nitrogen.

In 1924 Takahashi reported the isolation of vitamin A from cod liver oil in chemically pure form. He named the substance, "biostearin," and considered it an alcohol closely related to cholesterol. In 1925 Drummond, Channon, and Coward challenged Takahashi's claim, affirming that they had obtained equally active concentrates, with due regard to varying activity of the original oils, and that such concentrates were known to be highly impure, containing, in addition to vitamin A, a saturated alcohol, one or more unsaturated alcohols, and an unsaturated hydro-

carbon, spinacene. They found vitamin A to be volatile in superheated steam. Takahashi reported the effect of biostearin as proportional to its concentration in the diet up to 0.05% but considered it to have an unfavorable effect at a higher level of intake. Drummond and co-workers have often administered large amounts of highly active concentrates without observing any ill effect. They considered it possible that Takahashi's material contained traces of a toxic impurity.

The vitamin A content of foods that are good sources of this vitamin seems not to be appreciably affected by the canning process or by such exposure to heat and air as occurs in ordinary cooking. Butter heated 4 hours at 120°C. with air bubbling through during the whole period loses all protective properties associated with vitamin A, but no loss is apparent when milk is boiled for a few minutes, or when pasteurized or dried by modern methods. Butter exposed to air at 37°C. gradually loses its vitamin A. Vitamin A is stable at high temperatures in the absence of oxygen and is not destroyed when fats and oils containing it are saponified by alcoholic solution of potassium hydroxide.

Water Soluble B. (Vitamin B.)—This vitamin is generally called the antineuritic vitamin because its absence from the dietary causes a polyneuritis. It was in the study of a form of polyneuritis in man (beriberi) that our interest in this food essential was aroused. Deficiency or absence of this vitamin from the dietary leads not only to malnutrition of the nervous system but to disorders of digestion and malfunctioning of endocrine glands. The appetite is impaired, diarrhoea often produced and undermining of strength and vigor results. Injury done the alimentary tract is more lasting than are the lesions of nervous structures and is more resistant to treatment. Susceptibility to infectious diseases seems increased. The antineuritic vitamin has been supposed to play an important part in carbohydrate functioning.

Milk, eggs and the glandular organs contain this vitamin in relatively large amount. Meat muscle has it in less degree. It is present in sufficient amount in whole cereal grains but in the milling of such foods the embryo and the outer coverings of the seed, which chiefly contain this vitamin, are lost, and the remaining starch core has but little. Tomatoes form an excellent source of supply and most leafy vegetables provide it in sufficient amount. Yeast and germinating seeds are particularly rich in antineuritic vitamin and nuts are also good sources.

Yeast and other sources of the dietary factor, known up to the present time as vitamin B, contain, besides the water soluble anti-neuritic complex, another water soluble complex—the P.P. factor—and perhaps at least one other. Sherman has proposed that the anti-neuritic factor be renamed vitamin F and P.P. factor of Goldberger, vitamin G, thus supplanting the term, vitamin B. For historical reasons vitamin B will probably continue to be the designation for the anti-beriberi vitamin and P.P. factor for the anti-pellagra vitamin. Goldberger and co-workers have found that both the anti-neuritic and the pellagra preventive factor must be supplied to promote growth of rats. The water soluble factor regarded by different investigators as existing in yeast together with the anti-neuritic vitamin is, perhaps, the P.P. factor, not tested by them for anti-pellagrous activity. The existence of a third vitamin has not been established, but whatever the number, yeast fulfills the requirements for growth, prevention of beriberi and prevention of pellagra. As Steenbock has stated, all or several vitamins, fat soluble as well as water soluble,

are required for normal growth and it is not proper to designate any one as *the* growth promoting vitamin.

Seidell of the U. S. Hygienic Laboratory and Levene of the Rockefeller Institute have both succeeded in separating from brewers' yeast, preparations containing the so-called vitamin B, largely free from other organic matter. Funk and others in earlier years made concentrates. Seidell's latest mixtures are prepared from brewers' yeast by boiling and filtering. The vitamin B, also P.P. factor, and other water soluble factors if they exist, are removed from the solution by adsorption with fuller's earth or by precipitation. He has used pigeons for controlling his chemical procedures and it has thus naturally happened that the substances prepared by him are especially antineuritic for pigeons. Smith, in the same laboratory, found these preparations to have much less growth-promoting activity for rats than whole yeast. Levene, on the other hand, has used rats as controls and his concentrates are actively growth-promoting for those animals.

Different samples of dried yeast preparations sold commercially vary considerably with respect to content of the P.P. factor and the anti-neuritic factor. Concentrates from yeast are made by boiling and evaporating a water extract to a dry powder. A concentrated autolyzed brewers' yeast is also obtainable.

The principal amount of work published on vitamins has necessarily been based on the results of biological experiments. The limitations of the biological method should be borne in mind. Different species of animals differ so greatly with respect to vitamin requirements that the results of dietary experiments cannot be assumed to hold for other species. Fundamental knowledge of chemical structure is much needed; and, until such information is obtainable, progress in the study of vitamins must necessarily be slow and uncertain.

Vitamin B is affected less by exposure to oxygen than either A or C, and is not appreciably affected by the heat ordinarily applied in the processing of canned foods. Its stability is probably greater in some foods than in others; cereal seeds and legumes, for example. Yeast extract which is a particularly rich source loses practically all of its antineuritic properties when heated for 2 hours at 122°C., and the same is true of cereal seeds. Little or no loss results in cooking foods or in pasteurizing milk.

Water Soluble C. (Antiscorbutic Vitamin.)—In the absence of fresh fruits and vegetables, or of milk obtained from cows which have been fed on fresh feed, we are confronted with the danger of the development of scurvy; but fresh meat, particularly fresh glandular organs, or certain canned foods which have an acid reaction (as canned tomatoes), may provide a sufficient amount of the antiscorbutic vitamin. Dried peas or beans when made to germinate furnish a good source of vitamin C. It must be kept in mind that in addition to the frank manifestations of scurvy or infantile scurvy we must recognize certain vague manifestations of ill health and lowered resistance when a diet is on the borderline of a vitamin C deficiency. The rat is either able to synthetize this vitamin or avoid scorbutic manifestations without it. Hess has noted a borderline condition in infants, who, in the absence of typical infantile scurvy, show an irritability and lack of vigor which yield to orange or tomato juice.

Vitamin C tends to be destroyed quickly by oxidation, especially in the presence of heat, but certain acid foods may be subjected to prolonged heating in the absence

of air without appreciable loss of this vitamin, for example, canned tomatoes. Oxidation is the greatest destructive influence, a short exposure to high temperature causing less destruction than prolonged exposure to a moderate degree of heat. Milk a slightly acid food, may be subjected in the absence of air to the degree of heat required in "evaporating" and canning without noticeable destruction. Milk of course, is not a good source of vitamin C. In general, vegetables which in the raw state are fairly good sources lose to a great extent their power to protect against scurvy when cooked, hence, the importance of including in the diet the juices of citrus fruits, raw tomatoes, lettuce, and cabbage which may also be eaten raw as well as cooked. The prolonged boiling which accompanies the preparation of stews destroys all of vitamin C and may destroy a part of vitamin A.

The comparative values of different foods as sources of vitamins A, B, and C, based on the work of many investigators, are indicated in the following table. The values as set forth are not to be taken too literally. As pointed out by McCollum there is no adequate procedure for estimating quantitatively any vitamin. Unless this is borne in mind the table may be misleading.

Values:

\+ indicates that the food contains the vitamin but is not a good source.
++ indicates that the food is a good source.
+++ indicates that the food is an excellent source.
− indicates that the food does not contain the vitamin.
± indicates that it is doubtful if the food contains the vitamin.
? indicates a lack of experimental evidence.

VITAMINS IN FOOD

Classes of foodstuffs	Fat soluble A factor	Water soluble B or anti-neuritic (anti-beriberi) factor	Water soluble C or anti-scorbutic factor
Meat, fish, etc.:			
Lean meat (beef, mutton, etc.)	− to +	− to +	− to +
Liver	++	++	+
Kidneys	++	+	?
Heart	+	+	±
Brain	+	+	?
Sweetbreads	+	+	?
Fish, muscle, lean	− to +	− to +	?
Fish, muscle, fat (salmon, herring, etc.)	+	− to +	?
Fish, roe	+	+	?
Tinned meats	− to +	− to +	−
Oysters	++	++	+
Clams	++	+	?
Crabs	?	?	?

VITAMINS IN FOOD (*Continued*)

Classes of foodstuffs	Fat soluble A factor	Water soluble B or anti-neuritic (anti-beriberi) factor	Water soluble C or anti-scorbutic factor
Dairy products:			
Milk, cow's whole, raw	+++	++	+
Milk, dried whole	++	++	− to +
Milk, boiled whole	+++	++	−
Milk, condensed	++	++	− to +
Cream	+++	+	− to +
Cheese, whole milk	++	±	−
Cheese, skim milk	+	+	−
Eggs, fresh	++	+	±
Fats and oils:			
Butter	+++	−	−
Cod liver oil	+++	−	−
Mutton and beef fat or suet	+	−	−
Lard	±	−	−
Cottonseed oil	±	−	−
Olive oil	−	−	−
Peanut oil	−	−	−
Margarine prepared from animal fat	− to ++	−	−
Margarine from vegetable fats or lard	−	−	−
Nut butters	− to +	−	−
Vegetables:			
Leaves, stalks, flowers:			
Asparagus	?	+++	?
Cabbage, fresh, raw	+	++	+++
Cabbage, fresh, cooked	+	++	++
Cauliflower	+	++	++
Celery	− to +	++	?
Chard	++	+	?
Lettuce	++	++	++
Rhubarb	?	?	+
Spinach	+++	+++	++
Squash, Hubbard	+	?	?
Roots, bulbs, tubers:			
Beet root	−	+	− to +
Carrots, fresh raw	++	++	++
Onions, raw	?	+	++
Onions, boiled	?	+	− to +
Irish potatoes (cooked with skin)	− to +	+	+ to ++
Irish potatoes, raw	− to +	+	++
Sweet potatoes, cooked	++	+	+ to ++
Rutabaga	−	++	++
Cereals:			
Barley, oats, rye, whole grain	±	++	−
Wheat, maize (yellow), rice, whole grain	− to +	++	−
White wheaten flour, pure corn-flour, polished rice, etc	−	− to +	−
Germinated cereals	+	++	+

VITAMINS IN FOOD (*Continued*)

Classes of foodstuffs	Fat soluble A factor	Water soluble B or anti-neuritic (anti-beriberi) factor	Water soluble C or anti-scorbutic factor
Legumes:			
Beans, kidney	±	+++	−
Beans, Navy	+	+++	−
Beans, soy	+	+++	−
Peanuts	−	++	?
Peas, fresh	++	++	++
Germinated legumes	+	++	++
Fruits:			
Apples	+	+	++
Bananas	+	+	++
Eggplant	−	++	?
Grapefruit	−	++	++
Grape juice	±	+	+
Lemon juice, fresh	−	++	+++
Lime juice, fresh	−	+	+
Lime juice, preserved	−	?	− to +
Orange juice, fresh	+	++	+++
Raisins	−	+	−
Raspberries (fresh and boiled)	?	?	++
Tomatoes, fresh	++	+++	+++
Tomatoes, canned	++	+++	+++
Nuts:			
Almonds	+	++	?
Cocoanut	+	++	?
Pecans	+	++	?
Walnuts (English)	+	++	?
Sugar and starches:			
Honey	−	+	−
Sugar	−	−	−
Miscellaneous:			
Bread, white (water)	−	+	−
Bread, white (milk)	+	+	− to +
Yeast	?	+++	−
Meat extract	−	−	−
Beer	−	+	−
Alfalfa and clover	+++	++	?

Vitamin D (antirachitic factor).—In 1922, McCollum and coworkers discovered in cod liver oil a "calcium-depositing substance" distinct from vitamin A, and this has come to be regarded as a vitamin whose function it is to regulate the mineral metabolism of the bones and probably the teeth. Steenbock and others showed that irradiation of animals with ultraviolet light for 10 minutes daily in the absence of fat soluble vitamins in the diet increased both calcium and phosphorus in the blood. Experimental studies led to the inference that cod liver oil and also radiant energy

serve to maintain the normal salt equilibrium of the body in the presence of different combinations of salts as absorbed in foods.

Later it was found that various fats and oils of animal and vegetable origin, but not mineral oil, could be "activated" by exposure to sunlight or ultraviolet rays so that the activated food had the same qualitative effect, but not to the same degree, as cod liver oil in the prevention and cure of rickets experimentally induced in rats. A rat kept in the dark and fed a diet lacking in vitamin D, after developing rachitic lesions, and after being cured by irradiation with ultraviolet rays, was killed. Its liver was found to be curative for a rachitic rat. The liver from a rat which has not been irradiated is inactive. The activity of a liver taken from an irradiated rat was not destroyed by drying at 96°C. for 24 hours and keeping it in a stoppered bottle for 2 months.

Numerous investigators have been attracted to this field of research. Steenbock and his associates discovered that the unsaponifiable constituents of certain crude fats could be activated by irradiation with ultraviolet rays—presumably the sterols. It then appeared that recrystallized cholesterol could be activated. Later, Kramer and co-workers, Hess and others, reported that irradiated cholesterol could be separated into an active and inactive fraction. Activated fractions were removed, the remaining cholesterol was recrystallized and again irradiated with the repeated production of activated fractions in the form of yellowish oil or resin. These fractions when collected and mixed with an inactive oil such as cottonseed oil cured experimentally induced rickets in rats.

These studies led to investigations which have increased our knowledge of the chemical nature of the sterols. In 1927, Rosenheim and Webster in England described methods, physical as well as chemical, by which cholesterol and phytosterols can be so purified that they can no longer be rendered antirachitic by exposure to ultraviolet rays. They concluded that the activatable impurity is a sterol of an unsaturated and labile type. They worked with ergosterol which was first isolated from ergot by Tanret (1890–1908). That is a highly unsaturated sterol which apparently can be obtained from a wide variety of lower plants including certain yeasts. Ergosterol was found to possess the same characteristic absorption spectum in the ultraviolet range as non-purified cholesterol obtained from any ordinary animal or vegetable source, but the intensity of absorption was enormously greater. With regard to chemical structure, ergosterol ($C_{27}H_{42}O$) has three double bonds. After irradiation, ergosterol was found to prevent and cure rickets in rats on a rachitic diet in daily doses so very small as one ten-thousandth of a milligram. These results would seem to indicate that ergosterol is the most potent antirachitic substance yet identified. The investigators noted that a dose 10,000 times greater than that regarded as an effective dose, produced no obvious ill effects on rats.

After it was discovered that ordinary cholesterol could be sufficiently purified by chemical treatment with bromides, or by certain other means, to lose the power of becoming antirachitic by irradiation, it was concluded by these investigators that the precursor of Vitamin D (provitamin) is not ordinary cholesteral but a substance associated with cholesterol as obtained from all natural sources—a sterol having more than one double bond, identical with or similar to ergosterol. This, the provitamin or natural parent substance of Vitamin D is apparently converted into the vitamin by irradiation. It was estimated that the provitamin sterol is

present with cholesterol in the proportion of about 1 to 2000. The minute quantity concerned was thought to be the reason why its presence was not recognized before it became possible to apply a biological test.

Rosenheim and Webster found it necessary to irradiate a solution and not the crystals of ordinary colesterol in order to induce the complete destruction of both the "provitamin" and the vitamin. Incidentally they remarked that in view of the variable content of provitamin in commercial samples of cholesterol which may possibly have been partially purified by means of charcoal, caution is indicated against the indiscriminate use of irradiated cholesterol as a source of vitamin D, especially when testing articles of food for the presence of Vitamin A.

Unlike the vitamin obtained from it, the unsaturated sterol, assumed to be the provitamin, forms an addition compound with digitonin, but complete separation from cholesterol is not practicable and it is not surprising that erroneous conclusions were drawn from earlier work with cholesterol in which digitonin was largely relied upon in chemical treatment. The action of ultraviolet rays on ergosterol or the provitamin sterol leads to an obvious physical change and the production of a yellowish resin of manifestly great antirachitic power, but the nature of the intramolecular change which gives rise to the vitamin formation was not determined.

It thus appears that ultraviolet radiant energy is behind the production of the so-called vitamin D. Extensive application in the clinical treatment of rickets is being made of the knowledge experimentally derived. Cod liver oil is now widely used, often without the advice of a physician, and the effectiveness of sunshine in preventing and curing rickets is coming to be so generally appreciated that the disease presumably will rarely be seen in any part of the World by future generations.

Cod liver oil is at present the outstanding source of the antirachitic factor in form suitable for ingestion, but other sources equally or more potent may in the future be developed under the influence of ultraviolet rays. Hydroquinol, egg yolk, bile, and sperm oil have been reported to have some antirachitic power. It has also been reported that the substances curative of rickets upon oxidation blacken photographic plates through quartz but not through glass screens. Injection of hematoporphyrin has been found to cause deposition of calcium salts in rachitic rats. This substance renders them very sensitive to light and the animals are likely to die before the cure can be completed.

McCollum states that cod liver oil fed in amount representing 2% of the food ingested, rapidly cured experimentally induced rickets, whereas butter to the amount of 30% only gave evidence of beginning cure in 11 to 14 days. Cocoanut oil was less potent than butter, and other vegetable oils were found inert. Shark liver oil and burbot liver oil appeared to be as potent or nearly as potent as cod liver oil.

The experimental studies of Howland support the conclusion that vitamin D, direct exposure to sunshine, or irradiation with ultraviolet rays helps to bring about the end result (increased deposition of calcium and phosphorus with cure of rickets) by promoting absorption of these elements from the gastrointestinal tract. With deficiency of vitamin D, healing of rachitic lesions will not take place except in restricted border line cases where the ingestion of a slight excess of calcium may lead to sufficient absorption into the blood stream to cause deposition in the bones with a specially favorable calcium-phosphorus ratio. In general, however, the ingestion of an excess of calcium tends to cause precipitation of the comparatively insoluble

tricalcium phosphate in the intestine. Alkaline phosphates increase the excretion of calcium in the feces. Calcium as well as phosphorus absorption is reduced when the diet is poor in fat.

In human as well as in experimentally induced rickets, the concentration of calcium in the blood plasma is reduced. The same is true of inorganic phosphorus. Howland and Kramer found that the normal concentration of calcium in the serum of rats was 9.5 to 10.5 mgm. per 100 cc., and of phosphorus, 7 to 8.5 mgm. These figures give a *solubility product constant* of 66 to 89 (obtained by multiplying Ca concentration by P concentration). They found the calcium concentration in healthy infants to be 10 to 11 mgm. and the normal phosphorus concentration to average 6 mgm. in breast fed infants and 5.5 in those fed on cows' milk Howland found that when the product was below 30 rickets was invariably present. When above 40, he concluded either that demonstrable healing was taking place or that there had been no rachitic change. He usually found rickets present with products between 30 and 40 He considered that at the same temperature with given concentrations of calcium and phosphorus the precipitation of tricalcium phosphate depends largely upon the hydrogen-ion concentration. Precipitation occurs with decreased hydrogen-ion concentration. In the body tissues, varying concentration of carbon dioxide is an important factor. The precipitation and deposition of calcium are considered to take place in cartilage and bone because in those tissues little CO_2 is present. The concentration of inorganic phosphorus in the serum is regularly reduced in uncomplicated rickets, but it is impossible to consider phosphorus independent of calcium because each definitely influences the other. In general, a factor which influences the concentration of one similarly influences the other.

With an adequate intake of protein of good growth-promoting value there can hardly be a deficiency of phosphorus. On the other hand, diets of Americans tend to be deficient in calcium. Such deficiency cannot be carried below a certain point without interference with bone metabolism even when vitamin D is abundantly available. When there is a comparative deficiency of this factor an excess of either calcium or phosphorus in the food tends with relatively greater effect to prevent the absorption of sufficient calcium for the normal growth and repair of bone tissue. Practically, calcium will not be in excess unless added to the food in considerable amount from some other source. Theoretically, with a disproportionate amount of phosphorus in the food and with less than optimal amount of sunlight or vitamin D, some calcium salt may be advantageously added to the food. While the adult is not so much in danger of disturbed metabolism arising from calcium deficiency as the rapidly growing child, and rickets does not occur, there is, nevertheless, reason to believe that with too little exposure to sunshine out of doors and absolute deficiency of calcium, damage often results from too low a concentration of calcium in the blood. The concentration of calcium in the serum of the normal adult is 10.5 mgm. per 100 cc. and the concentration of inorganic phosphorus is about 3.75 mgm. Maxwell and Miles found in cases of osteomalacia among women in China, concentrations of 5.2 to 7.4 mgm. of calcium with 1.8 to 3.8 mgm. of phosphorus per 100 cc. of serum.

Vitamin D is much more stable than vitamin A. Cod liver oil, when heated with air bubbling through it sufficiently long to destroy vitamin A, still retains its antirachitic power. Cod liver oil, as well as oils activated by irradiation, loses its vita-

min D activity with prolonged exposure to ultraviolet rays. Exposure to daylight probably causes a gradual reduction in the antirachitic value of cod liver oil.

Vitamin E (Substance X)—Antisterility and Lactation Promoting Factor.—As early as 1920 several investigators observed that milk and bread seemed to be lacking quantitatively and qualitatively in substances necessary for adolescent growth and reproduction of rats. In 1922, Evans and Bishop announced the existence of a dietary factor essential for reproduction which they named, vitamin substance X, antisterility factor.

Sure, who has since contributed much information regarding this factor, proposed the name vitamin E, which has been generally accepted. It is a fat soluble vitamin. Oil extracted from wheat embryo has furnished excellent concentrates for experimental purposes. The vitamin is contained in the nonsaponifiable fraction of the oil.

Certain experiments have led Sure to believe that wheat embryo oil may contain two active substances rather than a single factor. One prevents sterility of both male and female rats. The other seems to be required in liberal amounts during the lactation period. Otherwise the mother rat's milk fails to nourish the young. With the factor very deficient, the young die, often shortly after birth; with it present in somewhat greater but still inadequate amount the young fail to grow at a normal rate, and many die late in the lactation period, few being successfully weaned within the normal time limit of 21 to 25 days.

At the present time, the term, vitamin E, includes both factors, if there be two. If the antisterility factor is lacking or deficient in quantity, female rats either do not become pregnant or resorption of the fetus takes place with autolysis, even the skeletal tissue going into solution. The placenta, which is last to be absorbed, shows blood extravasations in earlier stages of the process. Male rats reared on a diet lacking in the antisterility factor show degenerative changes in the testicles, the gross weight of which is less than normal, and the animals are sterile. There is a critical period of 90 to 150 days from the time of birth after which males who have been deprived of the factor cannot be made fertile by feeding the vitamin even in liberal amounts. In the case of females, the results of deprivation can apparently be overcome at any time during the reproductive period. With the vitamin present, but somewhat deficient in amount, females give birth to dead or emaciated young.

Lactation is undoubtedly affected by a number of dietary factors. It is essential that the mother animal's diet contain sufficient amounts of the growth promoting vitamins, fat soluble A and water soluble B, especially a liberal amount of the latter, as well as fat soluble D and minerals.

Sure, Evans, and Burr believe that females have considerable capacity for storing the antisterility factor, but recently performed experiments have led Sure to believe the female rat has at most a very limited capacity for storing the complex essential for successful mammary gland function.

For experimental purposes wheat embryo has so far been the outstanding source of vitamin E which may be extracted from that germ and from other sources with 80 to 95 per cent. alcohol, ether, acetone, or benzine. Prolonged extraction (4 to 36 hours) with the best fat solvents is required to extract all the oil from wheat embryo. A considerable amount of the vitamin may be left in the embryo if extracted for only a few hours. The following food stuffs have been found to contain considerable

quantities of vitamin E; lettuce leaves, alfalfa leaves, kale, whole wheat, yellow corn, rolled oats, Canadian field peas, hemp seed, Georgia velvet bean pod, egg yolk, beef and beef liver. Evans and Bishop stated that this substance is practically absent from milk, but recent work by Mattill and Clayton indicates that milk fat probably contains more vitamin E than previously estimated. It is not present in cod liver oil or in yeast in significant amount. Olive oil and also oils extracted from peach kernels, soy beans, and peanuts cured sterility but were not potent for lactation. Vitamin E appears to be lacking in linseed oil, commercial cocoanut oil, commercial corn oil (destroyed in processing) and sesame, palm kernel, rape seed, mustard seed, and sweet almond oils. McCollum and co-workers believe the fats of beef liver contain vitamin E.

The substance having antisterility properties, as contained in wheat oil, is stable to heat and aeration. Sure found that wheat oil retained its antisterility properties after autoclaving for 2 hours at 20 pounds pressure and also after aerating at room temperature for 72 hours. Lactation promoting properties were apparently destroyed by heat and by oxidation. He also observed that as little as 1 mg. of wheat germ oil per rat per day sufficed for fertility but not for successful lactation. The addition to a sterility producing diet of 0.03% by weight of the ration of the unsaponifiable matter from wheat germ oil, freed from sterol as much as possible, was sufficient for the birth and also for the successful rearing of normal rat litters. As little as 0.01% sufficed to prevent resorption. One milligram of such a concentrate per day prevented sterility but 4 milligrams permitted the successful rearing of 75% of the young rats born. With 2 milligrams, only 9 out of 60 were successfully weaned although healthy normal litters were born.

In 1927, Simmons, Becker, and McCollum reported experiments indicating that vitamin E is a substance which specifically affects the assimilation of iron. In view of such a relationship and that existing between the action of vitamin D and calcium-phosphorus metabolism the question is raised whether organic factors hitherto unrecognized are concerned in the utilization of other essential mineral substances. They observed, while investigating so-called salt ophthalmia, that their rats declined in weight and developed ophthalmia when ferrous sulphate was the source of iron in a mixture of purified salts supplying necessary mineral elements, even though the diet contained a sufficient amount of vitamin A. Male rats, apparently became sterile. Their testes became small and rather bluish, and spermatazoa disappeared. Under the conditions of the experiments it was not to be expected that the female rats would become pregnant but other experiments indicated that under similar conditions resorptions, such as those described by Evans and Bishop, would take place.

The rats recovered from ophthalmia and enfeeblement when ferric citrate was fed instead of ferrous sulphate. They also recovered with the latter as the sole source of iron, provided wheat embryo oil was added to the diet. Moreover, a small amount of that oil in the diet was sufficient to maintain fertility regardless of which iron salt was fed. The function of vitamin E thus appeared to be associated in some manner with the assimilation of iron, and the death of fetuses in rats on a diet deficient in the vitamin seemed to result from a crisis in iron assimilation. As a source of iron for growing rats, ferric salts were regarded as far superior to any ferrous salts examined.

The results of these experiments led the investigators to question the wisdom of using ferrous sulphate as a therapeutic agent in the practice of medicine. To them it would seem more logical to treat anemia with ferric citrate and a fat which is a source of vitamin E. They suggested that since beef liver contains iron, and its fats contain the vitamin, there is a possible explanation for the value of liver in the treatment of pernicious anemia as observed by Minot and Murphy. In view of the relationship thought by them to exist between the action of vitamin E and the assimilation of iron, deductions drawn from earlier experiments regarding dietary factors influencing fertility may require modification.

In this connection the results of certain experiments reported by Hart and coworkers in 1927 are of interest. Rabbits exclusively fed on whole milk develop anemia. Anemia was not prevented by adding ferric oxide to the diet or by adding purified ferrous sulphate, although impure ferrous sulphate appeared to be an effective agent in the prevention and cure of nutritional anemia in their animals. The addition of either fresh cabbage, or an alcoholic extract of dried cabbage or lettuce or an alcoholic extract of cornmeal to the whole milk and ferric oxide diet, supplied a factor necessary for the prevention and cure of the anemia. This was at first thought to be an organic factor but later it was found that the ash of 5 grams of lettuce or the ash of 5 grams of dried cabbage, after heating to 650°C. and higher, was an equally effective supplement. The ash contained no organic matter but did contain traces of iron. These investigators concluded that the deficiency in a milk-ferric oxide diet leading to nutritional anemia in their animals was of inorganic rather than of organic nature. The whole milk, presumably, furnished enough vitamin E to meet the growth and repair requirements of the rabbits. Fertility was not tested.

Pellagra Preventive Factor (P.P. Factor of Goldberger).—In 1925 Goldberger and Tanner announced the existence of a hitherto unrecognized or unappreciated factor necessary in the diet to prevent or cure pellagra. In foods which have been found to have anti-pellagrous activity the P.P. factor is associated with the antineuritic factor which has been synonymous with the term, vitamin B. Sherman suggested the designation, vitamin G, for the factor discoverd by Goldberger and co-workers. See remarks under vitamin B.

Yeast is at present the richest known source of the P.P. factor, as it is of the antineuritic complex. Both are water soluble. Antipellagrous activity of yeast or of watery extracts thereof is not materially affected by autoclaving for two and one-half hour at 15 pounds pressure, although that treatment results in nearly complete destruction of the antineuritic vitamin. The P.P. factor is partially or largely separated from other organic matter by treating a water extract of autoclaved yeast with fuller's earth which, thus activated, is known as P.P. solid. This may be used in the treatment of pellagra but it is prepared especially for research purposes and has no advantage over the original yeast in clinical practice.

Goldberger has determined the presence of P.P. factor in certain foods studied by him and his associates by noting the activity of each in preventing pellagra among significant groups of pellagrins and also by determining the effectiveness of each to prevent black tongue in dogs. Black tongue, which can be experimentally produced following a period of dietary deficiency, is a condition which Goldberger considers the analogue of pellagra in human beings.

Yeast, fresh or dried, is so far, the most potent article that can be added to a pellagrin's diet. By actual trial, fresh beef and milk have been found efficient in the prevention and treatment of pellagra when fed daily in liberal amounts. Although canned tomatoes appeared to be deficient or lacking in P.P. factor when fed daily to pellagrins in amounts equivalent to those that would ordinarily be ingested in a mixed diet, the daily ingestion of a quart of canned tomatoes has recently been found to prevent pellagra.

The P.P. factor is probably present in all foods that contain the antineuritic vitamin, but quantitatively it does not necessarily parallel antineuritic potency. A given source may be more or less potent in this respect. For example, some strains of yeast which appeared to be unusually poor in antineuritic complex, according to usual tests with animals, were nevertheless found to be potent sources of P.P. factor. The converse may also be true.

Composition of Ordinary Food Materials

(From Lusk, after Atwater and Bryant)

Kind of food material	Inedible refuse of purchased material, %	Edible portion						
		Water, %	Unavailable nutrients, %	Available nutrients				
				Protein, %	Fat, %	Carbohydrates, %	Ash, %	Fuel value per lb. = 453.6 grams, calories
Beef (fresh):								
Ribs	20.8	55.5	2.0	17.0	25.3	.....	0.7	1430
Round, lean	8.1	70.0	1.0	20.7	7.5	.....	1.1	735
Hind quarter	15.7	59.8	1.8	17.8	20.5	.....	0.7	1240
Beef (preserved and cooked):								
Canned, corned	....	51.8	2.7	25.5	17.8	.....	3.0	1275
Roast, cooked	....	48.2	2.4	21.6	27.2	.....	1.0	1410
Veal (fresh):								
Hind quarter	20.7	70.9	1.2	20.1	7.9	.....	0.8	740
Liver	....	73.0	0.9	9.7	5.0	.....	1.0	410
Lamb (cooked):								
Chops, broiled	13.5	47.6	2.5	20.1	28.4	.....	1.4	1640
Leg, roast	....	67.1	1.4	19.1	12.1	.....	0.6	905
Mutton (cooked):								
Leg, roast	....	50.9	2.1	24.3	21.5	.....	0.9	1410
Pork (pickled, salted and smoked):								
Ham	13.6	40.3	3.6	15.8	36.9	.....	3.6	1905
Pork (cooked):								
Ribs, cooked	....	33.6	3.1	24.1	35.7	.....	1.7	2020
Poultry and game (fresh):								
Fowl	25.9	63.7	1.6	18.7	15.5	.....	0.8	1040
Fish (fresh):								
Cod steaks	9.2	79.7	0.9	18.1	0.5	.....	0.9	385
Mackerel	44.7	73.4	1.3	18.1	6.7	.....	0.9	650
Shell-fish (fresh):								
Oysters, in shell	81.4	86.9	0.8	6.0	1.1	3.7	1.5	235
Fish (preserved and canned):								
Cod, salt, boneless	1.6	55.0	5.5	24.9	0.3	.....	14.3	510
Salmon, canned	14.2	63.5	1.9	21.1	11.5	.....	2.0	915
Eggs:								
Eggs, boiled	11.2	73.2	1.2	12.8	11.4	.....	0.6	755

COMPOSITION OF ORDINARY FOOD MATERIALS (*Continued*)

Kind of food material	Inedible refuse of purchased material, %	Edible portion						
		Water, %	Unavailable nutrients, %	Available nutrients				
				Protein, %	Fat, %	Carbohydrates, %	Ash, %	Fuel value per lb. = 453.6 grams, calories
Dairy products, etc.:								
Whole milk	....	87.0	0.5	3.2	3.8	5.0	0.5	310
Skimmed milk	....	90.5	0.3	3.3	0.3	5.1	0.5	170
Cheese	....	34.2	3.4	25.1	32.0	2.4	2.9	1885
Butter	....	11.0	4.9	1.0	80.8	.....	2.3	3410
Miscellaneous:								
Gelatin	....	13.6	3.2	88.7	0.1	.....	1.6	2125
Cereals, etc.:								
Corn (maize) meal	....	12.5	4.0	7.5	1.7	73.5	0.8	1625
Oatmeal, boiled	....	84.5	0.9	2.3	0.5	11.3	0.5	285
Rice, boiled	....	72.5	1.1	2.3	0.1	23.8	0.2	505
Gluten flour	....	12.0	4.6	11.0	1.6	70.1	0.7	1630
Wheat flour:								
Family and straight grade	....	12.8	4.0	8.3	1.0	73.5	0.4	1615
Bread:								
Corn (johnnycake)	....	38.9	3.5	6.5	4.2	45.2	1.7	1170
White wheat	....	35.3	3.3	7.1	1.2	52.3	0.8	1195
Sugar, starches, etc.:								
Sugar, granulated	....	....	...	....	....	100.00	...	1790
Vegetables:								
Asparagus, fresh	....	94.0	0.7	1.3	0.2	3.3	0.5	95
Beans, lima, green	55.0	68.5	2.7	5.3	0.6	21.6	1.3	525
Beans, string, cooked*	....	95.3	0.5	0.6	1.0	1.9	0.7	90
Beans, baked	....	68.9	2.8	4.8	2.3	19.6	1.6	565
Beets, cooked	....	88.6	1.2	1.7	0.1	7.2	1.2	170
Cabbage	15.0	91.5	0.7	1.2	0.3	5.5	0.8	140
Carrots, fresh	20.0	88.2	1.0	0.7	0.4	8.9	0.8	200
Sweet corn, green	61.0	75.4	1.8	2.3	1.0	19.0	0.5	445
Lettuce	15.0	94.7	0.5	0.9	0.3	2.9	0.7	85
Peas, green, cooked*	....	73.8	2.5	5.1	3.1	14.4	1.1	490
Potatoes, cooked boiled	....	75.5	1.7	1.9	0.1	20.0	0.8	415
Spinach, cooked*	....	89.8	1.1	1.6	3.7	2.7	1.1	235
Sweet potatoes, cooked*	....	51.9	3.0	2.2	1.9	40.3	0.7	885
Turnips	30.0	89.6	0.8	1.0	0.2	7.8	0.6	175
Vegetables (canned):								
Beans, string	....	93.7	0.7	0.8	0.1	3.7	1.0	90
Sweet corn	....	76.1	1.7	2.1	1.1	18.3	0.7	430
Peas, green	....	85.3	1.4	2.7	0.2	9.6	0.8	235
Tomatoes	....	94.0	0.5	0.9	0.2	3.9	0.5	100
Fruits, etc. (fresh):								
Apples	25.0	84.6	1.6	0.3	0.5	12.8	0.2	260
Bananas	35.0	75.3	2.7	1.0	0.5	19.9	0.6	400
Blackberries	....	86.3	1.5	1.0	0.9	9.9	0.4	235
Grapes	25.0	77.4	2.4	1.1	1.4	17.3	0.4	390
Oranges	27.0	86.9	1.4	0.6	0.2	10.5	0.4	210
Pears	10.0	84.4	1.7	0.5	0.4	12.7	0.3	255
Fruits, etc. (dried):								
Dates	10.0	15.4	8.8	1.6	2.5	70.7	1.0	1415
Figs	....	18.8	8.7	3.4	0.3	67.0	1.8	1290
Raisins	10.0	14.6	9.1	2.0	3.0	68.7	2.6	1410
Prunes	15.0	22.3	8.3	1.6	....	66.1	1.7	1230
Fruits, etc. (canned):								
Peaches	....	88.1	1.3	0.5	0.1	9.8	0.2	190
Pears	....	81.1	1.9	0.3	0.3	16.2	0.2	310
Nuts:								
Almonds	45.0	4.8	10.9	17.8	49.4	15.6	1.5	2685
Cocoanuts	49.0	14.1	9.2	4.8	45.5	25.1	1.3	2460
Peanuts	25.0	9.2	10.7	21.9	34.7	22.0	1.5	2255

* With butter, etc., added.

Food Deficiency Diseases

Avitaminoses.—Diseases resulting from dietaries deficient in one or more of the vitamins are often called avtaminoses.

Many medical men have been identified with studies of the connection between diet and disease. Among them are Eijkman and Fraser and Stanton. Others were Backstrom who as early as 1703 was familiar with an antiscorbutic principle in food, and Lind (1772) and Trotter (1792) who clearly refer to the existence of such a principle. These medical investigators all worked with the best experimental animal—man.

It has of course always been known that ill health or death may result from an insufficiency of food, as during periods of famine or siege, but quite recent is the conception that disease due to lack of peculiar food elements may develop in persons receiving a diet providing adequate calories. Not until 1910 was it generally appreciated that proteins of high biological value, mineral constituents and "vitamins" are as necessary for the maintenance of health as substances possessing fuel value. The diseases which are now recognized as connected with these specific food deficiencies are the following: Rickets, scurvy, infantile scurvy, xerophthalmia, beriberi, infantile beriberi, nutritional oedema and pellagra.

It should be remembered that a diet which is lacking in one dietary essential is very likely deficient in other factors as well. While diagnosis of the diseases mentioned in the preceding paragraph may be comparatively easy when one is confronted with a typical clinical picture, yet there are so many borderline cases and so many overlapping signs and symptoms that the recognition of the particular type of disease presented by a patient may be difficult.

There may also be amino acid and mineral deficiency together with vitamin deficiency. The diagnostic problem may be further complicated by the presence of still other factors of morbidity which may be contributory or even of equal importance. An example is the presence of various infectious processes which develop in a system of lowered resistance.

It is to be noted that not all diseases characterized by faulty metabolism and disturbances of nutrition belong to the category of food deficiency diseases. Some pathologic condition of the alimentary tract, or animal parasite infection, may prevent proper assimilation of an entirely satisfactory diet; and such diseases as diabetes, hyperthyroidism, hypothyroidism, dyspituitarism and the like may develop when the diet is well balanced and adequate, they being primarily due to disorders of endocrine function. It may be that, in certain of the true avitaminoses, disorders of the glands of internal secretion, although secondary to the nutritional deficiency, play an important part in determining the symptom-complex.

Xerophthalmia and Night Blindness.—Both of these conditions are quite common in certain parts of the tropics, and the view prevailed that the heat of the tropics and the tropical sunlight were potent factors until our study of vitamin requirements showed the etiology to rest in deficiency of vitamin A. Rats fed on a diet lacking in this substance develop xerophthalmia.

Night blindness (nyctalopia) is best known among the crews of sailing ships, especially when becalmed in tropical waters, and some influence of sunlight was considered the cause; but we now appreciate that it is among such personnel that ship beriberi and scurvy are prone to occur by reason of deficiencies of vitamins B

and C. Under those circumstances the diet might also contain too little vitamin A. In those parts of the tropics where famine conditions are common, both xerophthalmia and night blindness are frequent in young children, not necessarily associated but frequently combined.

In a mild case of xerophthalmia there is a dry area of triangular shape, extending from either side of the cornea, covered with a fine, whitish foam. In severe cases the whole eye may be dry, wrinkled and opaque, and eventually the cornea may slough away and bring about destruction of the eye. There is very little pain attending this frightful condition. It appears that the first recognizable pathological change in xerophthalmia is that the lachrymal glands cease to function. Deprived of tears, the cornea and conjunctival sac become dry and yellow, in appearance resembling skin. Ulcerations and infections are probably secondary to the altered state of the tissues. The salivary glands react similarly to a deficiency in vitamin A, and resulting dryness of the mouth and throat may cause aphonia.

Elliot notes that from the earliest times it was the custom in China to treat these conditions with extracts of liver. We now know that liver and kidneys are glandular organs rich in vitamin A, although not so rich as cod-liver oil which is our best preventive and curative agent for these conditions.

Beriberi.—This most important disease of the Orient is, from a pathological point of view, similar to peripheral neuritis due to other causes except that in addition to the lesions of the peripheral nerves there is marked involvement of the pneumogastric, so that associated with the atrophies of the muscles of the extremities there is degeneration of the cardiac muscle.

The remarkable studies of Eijkman, previously mentioned, dealing with the type of peripheral neuritis developing in fowls fed on a diet of polished rice, established the fact that similar effects were produced in prisoners by a similar diet, whereas those given rice that had not been subjected to excessive milling remained free from beriberi. Some years prior to Eijkman's investigations, Takaki associated the prevalence of beriberi in the Japanese Navy with protein deficiency and by increasing the amount of proteins in the ration caused beriberi largely to disappear among the naval personnel. Strong in the Philippines demonstrated that beriberi was not an infectious disease but could be induced by feeding experiments along the lines laid down by Eijkman.

The work of these pioneers was to a great extent lost sight of owing to the then prevailing absorption of men's minds in the subject of the bacterial causation of disease, and medical attention was occupied almost exclusively with numerous claims of new discoveries in that field until 1910 when Fraser and Stanton demonstrated that coolies fed on polished rice frequently developed beriberi, while those given unpolished rice remained well. The essential difference between the two grades of rice is that in the milling of the grain the pericarp and the aleurone layers are rubbed off and thus there are lost in the "polishings" much of the protein and practically all of the antineuritic vitamin. Subjecting corn or wheat to the same process produces a similar result so that there is left only a core of starch with merely traces of protein and vitamin. The embryo also is lost in the milling and with it most of the vitamin A.

Symptomatology.—Muscular weakness, especially of the legs, and cardiac palpitation are the earliest manifestations of the disease. At about the same time,

hyperaesthesia of the calf muscles with blunting of sensation in hands and feet becomes noticeable. Oedema over the tibiae can be demonstrated. As the disease progresses, foot-drop, wrist-drop and general muscular atrophy may appear. The picture of muscular wasting and paralysis may dominate the case, constituting that type of the disease known as dry or paraplegic beriberi. Other cases with marked dilatation of the heart may show pulmonary oedema and pronounced anasarca—the so-called wet or dropsical beriberi. These cases give a very high mortality (50%).

Etiology and treatment.—It is now recognized that the disease is caused by a deficiency of the antineuritic vitamin, vitamin B. The ingestion of a diet deficient in this substance for a period approximating three months is required for the development of the peripheral neuritis. McCarrison has noted that along with the nerve involvement we have hypertrophy of the adrenals and atrophy of almost all other glands of internal secretion and that it is probable that some of the clinical manifestations of the disease, such as the oedema, may be due to endocrine dysfunction.

Milk is a good source of vitamin B and there is little loss by pasteurization or other thermal methods of preservation. Milk is also a valuable constituent of the diet because of its mineral content and the high biological value of its proteins. Eggs and the glandular organs of animals are also good sources of vitamin B and likewise most vegetables. In addition to the food—yeast or a concentrated preparation of yeast should be administered.

Infantile Beriberi.—This is a variety of beriberi, encountered among nursing infants, which would be difficult to recognize if one were unaware of the type.

In 1898 Hirota first noted in infants suckled by beriberic mothers the existence of a condition which more recently has been carefully studied by McLaughlin and Andrews and to which the name infantile beriberi is now generally given. In the Philippines it is called "taon." Clinically there are restlessness, vomiting, altered voice, increased heart action, oedema and cyanosis. After death there are found marked hypertrophy and dilatation of the right side of the heart with no change of the left side. The peripheral nerves show the lesions of beriberi of adults but in less severe form.

The disease most often declares itself acutely. The child is rather suddenly seized with great pain, cries constantly, and soon becomes cyanosed. Death, which may occur in a few minutes, or hours, is often thought to be due to meningitis, although there are no fever and no true convulsions—there being only rigidity of the body. Less frequently the disease appears in a chronic form in which vomiting and constipation are the prominent symptoms. There is often to be obtained from the mother, who herself may have only a rudimentary beriberi, the history of the loss of several children from this disease.

The infants improve rapidly when suitable diet is substituted for the mother's milk. An extract of rice polishings gives striking results in these cases.

Ship Beriberi.—This, a disease of importance on Scandinavian sailing ships, has derived its name from its resemblance to beriberi. Its main features are oedema of the lower extremities at times generalized, dyspnoea, cardiac palpitation and the possible termination by death as the result of acute cardiac paralysis. The striking point of difference is the absence of manifestations of neuritis, an observation generally made in clinical reports and confirmed by Nocht who in an autopsy of a case failed to find evidence of degeneration of the peripheral nerves.

Another point of distinction is that once the ship arrives in port and a diet of fresh meat and vegetables is substituted for the sterilized canned meats and desiccated and preserved vegetables previously ingested, the patient recovers rapidly so that in one or two weeks there remains no sign of the disease; whereas, although beriberics improve at once when put on a curative diet, the damage done the peripheral nerves makes complete recovery a matter of weeks or months. Nocht is of the opinion that ship beriberi is closely related to scurvy since he found sore gums and haemorrhages into muscles in some of his cases, and, he notes, even in true scurvy there may be dropsy without spongy gums and haemorrhages. Dropsy and soreness of the gums are not infrequently observed in a beriberi-like affection developing among men of the French fishing fleet off the Newfoundland banks.

Basset-Smith notes that close affinity of scurvy with other diseases resulting from deficient food supply has been demonstrated year after year. He says that from true scurvey we pass in one direction to ship scurvy, Rand scurvy and beriberi, while in the other direction we find infantile scurvy merging into rickets.

It is probable that the disease is due to a deficiency of vitamin B in a diet which is also deficient in other vitamins which have been destroyed by the processes of sterilization, canning or desiccation used to preserve foods for use at sea. Deficiency of essential minerals especially calcium may also be a factor.

Scurvy.—This disease was the scourge of the crews of the ships engaged in the voyages of exploration two or three hundred years ago, and during times of war, from the period of the Crusades through our War of Independence and the Crimea, scurvy has exacted a heavy toll. Even in the recent war it was an important disease, though more so in the civil population than among soldiers. Its importance in history is suggested by the statement, sometimes made, that scurvy more than any other single factor has determined the outcome of national conflicts. It is interesting to note that records of past centuries, especially such documents as the story of Captain Cook's voyages, show that the influence of fresh food in the prevention and cure of the disease was clearly recognized even then.

Symptomatology.—The onset of scurvy is insidious, there being at first only vague manifestations of ill health such as weakness, dizziness, irritability, cardiac palpitation and anaemia. The earliest of the signs definitely indicative of the disease are sponginess and bleeding of the gums, and fetor of the breath. The tendency to capillary haemorrhage, so characteristic of the disease, is noted also in other parts of the body. Petechial spots about the hair follicles of the skin are common, leading at times to a diagnosis of purpura. Haemorrhages into the mucous membranes occur at about the same time; subperiosteal haematomata or nodes often develop; brawny indurations of the subcutaneous tissues are particularly characteristic; slight injuries are attended by large ecchymoses, and epistaxis and haematuria are common features. The subperiosteal nodes tend to break down and form ulcers. The ankles are swollen. The temperature is usually subnormal in uncomplicated cases.

Etiology and treatment.—About the middle of the 18th century Lind, in England, recognized that lemon juice and orange juice would prevent and cure scurvy. After Lind's time lime juice came into use and was generally regarded as a specific. It is, of course, known now that orange juice and lemon juice are much superior to lime juice and that the latter may contain little or no antiscorbutic vitamin even in the

fresh state. Therefore, it is not surprising that the experiences of Captain Scott in the Antarctic and of others showed that lime juice preserved in bottles has little or no value in warding off scurvy. The more potent orange and lemon juices lose the vitamin when preserved by ordinary methods. Either may be concentrated to a syrupy consistency or even dried to a powder in a partial vacuum at low temperature with very little destruction of vitamin C. Tomato paste prepared by concentrating under similar conditions is also a reliable source of the vitamin. Milk at best contains only a moderate amount of vitamin C and may contain very little unless the cows have been fed on green fodder. Cabbage eaten raw is a good source but when cooked by boiling for twenty minutes loses more than three-fourths of its vitamin content. Germinating peas and lentils are fairly good sources, about equal to potatoes in this respect, but fresh foods such as fresh orange and lemon juice, swedes andtom atoes, constitute our best sources of the antiscorbutic vitamin.

Fresh liver is a good source of vitamin C as well as of A and B. Lean meat has little if any antiscorbutic activity.

Of all the known vitamins, vitamin C is the one most readily affected by heat or oxidation. The promoters of "proprietary" vitamins seem to have had even less success in the preservation of vitamin C than with the others, their products being almost entirely lacking in antiscorbutic power. As Hess has remarked, "Vitamins should be obtained from the dairy, the grocery and the market—not from the drug store."

Infantile Scurvy.—In infantile scurvy there is a tendency to separation of the epiphyses from the shafts of the bones together with extreme sensitiveness to any movement, particularly of the legs. The chief lesion is subperiosteal extravasation of blood. A markedly anaemic and asthenic condition is also characteristic.

The disease is likely to follow the use of sterilized milk instead of fresh milk unless vitamin C is provided from some other source. The addition of orange juice to the diet of every infant as early as it can be tolerated is indicated. Tomato juice may be substituted.

Rand Scurvy.—In investigating the endemic scurvy on the Rand, in South Africa, Darling noted as characteristic exaggeration of knee-jerks, hypertrophy and dilatation of the right heart and often vagal degeneration. Pathologically these cases were closely related to beriberi, but clinically they differed in showing spongy gums and a tendency to haemorrhage.

Rickets.—This is essentially a disease of infants during the period of dentition and before they begin to walk, but it appears from reports emanating from Central Europe, that since the war it has been not infrequent in children up to five to six years of age. Digestive disturbances and fretfulness or apathy usually precede the more characteristic manifestations of the disease which are (1) a diffuse soreness of the body, (2) slight fever with nocturnal restlessness and (3) profuse sweating, especially about the head and neck.

Symptomatology.—Children with rickets do not begin to crawl or walk as early as they should, and, even when walking has been established, they are disinclined to move about. The weakness of the musculature causes a pot-belly. The skull and the thorax are the first bony structures to show changes. During the fourth or fifth month the flat bones of the skull are thin and soft (craniotabes), and later on they show thickenings, causing protuberance of the frontal and parietal bones. The fontanelles are slow to close. The ribs become enlarged at the costochondral

junction (rachitic rosary) and depressed at the attachment of the diaphragm. Thickening of the epiphyses proximal to the wrists and ankles is very characteristic, and the legs and arms become curved and otherwise deformed—bow-legs, knock-knees, saber-shin, etc. Dentition is delayed and the teeth when erupted are defective.

Etiology.—For a time after the importance of the vitamins became known it was thought that rickets was due to a deficiency in vitamin A, but the present view is that it is connected with an unsuitable calcium-phosphorus ratio, or a deficiency in the more recently recognized anti-rachitic vitamin. See remarks relating to calcium and phosphorus metabolism under vitamin D. The same factors which are operative in the causation of rickets seem equally important in the matter of dental defects. Possibly the magnesium content of the food may also be a factor both in rickets and dental caries as Howe has found the magnesium content of decayed teeth to be about twice as great as in normal teeth; that is, there may be displacement of the essential calcium. In connection with the feeding of infants it is to be noted that cow's milk has a much greater content of magnesium than has human milk.

Treatment.—It now appears that success in the prevention and cure of rickets depends upon adequate exposure of the infant or growing child to sunshine out of doors, or in the absence of sunshine, upon the ingestion of cod liver oil as a source of vitamin D, coupled with the feeding of liberal quantities of milk or other good sources of calcium such as leafy vegetables. There is an increasing tendency to recommend that even healthy infants be fed cod liver oil during the months which have little sunshine. A quart of milk a day in the diet is desirable.

Nutritional Oedema.—In those areas of Europe where famine conditions were approached during the late war, there was noted by many observers a condition characterized by weakness and oedema to which various designations were applied, such as war dropsy, war oedema, etc.

The oedema was more pronounced than in ordinary cases of starvation, so there must have been operative causes other than simple lack of sufficient nutriment. The factors usually ascribed are hard work performed on a diet of low protein content and the consumption of large amounts of water and salt in the thin soups so prominent in the dietary. A low caloric supply also is probably important etiologically since a feature of the affection is the extreme depletion of the body in all nutritive reserves. The oedema was most common in the feet and legs, at times extending to the thighs and trunk, and in about one-half the cases involving the face. Marked muscular weakness and alimentary tract disturbances were common. On slight exertion there was dyspnoea and a slow pulse, but cardiac disturbances were not features of the disease. The urine was pale, of low specific gravity and free of albumin. There was reduction of red cells and a tendency to leukopenia. Upon disappearance of the dropsy, marked emaciation was apparent. As is well known, shortage of fats was marked in Central Europe so that, as was to be expected, ocular manifestations due to deficiency of fat soluble A were frequently noted. The cases of nutritional oedema tended to recovery under proper diet and hospital care.

In certain countries, as Java and Haiti, a form of nutritional oedema is prevalent among individuals whose diet, for economic or other reasons, is inadequate in one or more respects; and among infants, fed on a preponderatingly starch diet for long periods, there are observed most striking degrees of dropsy apparently similar

in all respects to famine dropsy. This form of oedema may be related also to that seen in beriberi, pernicious anaemia and cachexia. Again it may be emphasized that diverse clinical manifestations may result from dietaries that are not only deficient in caloric value, but also in mineral substances and more than one vitamin.

Pellagra.—This is a disease with a marked tendency to recurrence; but an attack may run a rather acute course with a rapidly fatal termination. The trend of symptoms is: (1) Mild neurasthenic manifestations in the winter, to be followed in spring by (2) disturbances of the alimentary tract consisting of stomatitis, burning sensations going up the oesophagus, gastric eructations and recurring diarrhoeas. (3) In addition to the neurological and alimentary tract disturbances we have the third and diagnostically the most important group of symptoms in the cutaneous system. The pellagrous eruption is characterized by strikingly symmetrical, sharply delimited patches of erythema resembling sunburn. The sites of preference are backs of hands, extending up forearms, ridge of nose or neck. The neurasthenia tends to pass into a toxic psychosis or even a confusional insanity.

Etiology.—The weight of experimental evidence supports the view that pellagra results from a lack or deficiency of the water soluble vitamin discovered by Goldberger and co-workers, named P.P. factor. The diet of a pellagrin ordinarily becomes progressively more deficient in the pellagra-preventive factor as the winter progresses. By spring, but before natural foods supplying at least a little of the antipellagra vitamin find their way into the diet, the symptoms have become more intense.

Casal, who is credited with having given in 1735 the first definite description of the disease, makes note in his writings of the preponderance of maize and the infrequent appearance of fresh meat in the diet of the peasants of the Asturias. In 1810 Marzari remarked on the relation of the consumption of maize to pellagra and Alpine scurvy—a local name for pellagra. Lombroso (1872 to 1909) elaborated the maize etiology and for many years insisted that maize was the prime factor in the causation of the disease.

For a time it seemed as if the older idea that pellagra was connected with a dietary defect, chiefly as regarded some factor present in a diet consisting largely of maize, had been replaced by one assigning as cause some infectious process, probably protozoal, possibly bacterial. MacNeal and his colleagues on the Thompson-McFadden Pellagra Commission, as well as other authorities on this disease, insisted on a probable infectious agent as cause; and later Jobling and Arnold investigated certain strains of fungi recovered from stools of pellagrins which produced a fluorescent substance. The fungus incriminated apparently belonged to the *Aspergillus glaucus-repens* group. It is interesting to note that various Italian investigators suggested that certain species of *Aspergillus* growing on mouldy corn were etiologically related to pellagra. There was also much study of the connection between photodynamic substances in the circulation and the symptoms of pellagra.

In recent years the most important work done in the study of pellagra from the point of view that it is a food deficiency disease is that of Goldberger.

In February, 1915, Goldberger started a "pellagra squad," consisting of 11 prisoners on a diet of wheat flour (patent), cornmeal, corn grits, corn starch, polished rice, granulated sugar, cane syrup, sweet potatoes, fat fried out of salt pork, cabbage, collards, turnip greens and coffee. Baking powder was used for making biscuits and corn bread. The food value of each man's diet averaged 2952 calories.

A control was carried out with prisoners on a normal diet. The experiment was continued until Oct. 31, 1915. Of the 11 volunteers on the excessive carbohydrate diet six developed symptoms. Loss of weight and strength and mild neurasthenia were early symptoms. Definite cutaneous manifestations appeared only after five months. The skin lesions were first noted on the scrotum, later appearing on backs of hands in two cases and back of neck in one case.

Sixteen volunteers, working under Goldberger, tried to infect themselves with blood, nasopharyngeal secretions, epidermal scales, faeces and urine from pellagrins. Various atria of infection were tried according to material; blood by intramuscular injection, excreta by mouth. After a period of six months all the subjects of the experiments remained well. This evidence is certainly against the infectious nature of the disease.

Before the discovery of the P.P. factor Goldberger and Tanner noted from a series of experiments there seemed to be no relation between pellagra and deficiency in either mineral salts or known vitamins, and that a low biological protein value is not necessarily indicative of a pellagra-inducing diet. While studying the nature of low protein biological values they were led to the opinion that it rested in certain amino acid deficiencies, and it was observed that certain amino acids were notably lacking in the proteins commonly consumed by pellagrins. It will be remembered that it is a question if zein, the principal protein of maize, contains any cystine. Acting on their observations, they administered cystine to two cases of pellagra with apparent benefit, and administered both cystine and tryptophane to a third case with resulting steady improvement. It still appears that the progress of the disease or the promptness of recovery depends somewhat upon a sufficient intake of proteins of good biological value.

Treatment.—It follows from what has been said that the required vitamin should be furnished in liberal amount. The diet should include liberal quantities of beef and milk. In general the aim should be to provide a well balanced diet of sufficient fuel value. The diet may be supplemented with yeast or an active preparation of yeast.

Q—DISEASES FOLLOWING INGESTION OF TOXIC PLANTS

1. Atriplicism.—A disease of North China supposed to be due to the toxic properties of a weed, *Atriplex littoralis*, which grows in gardens around Pekin, but possibly caused by a small insect often found on the weed, as it is claimed the weed will not cause disease if well washed before eating. It is only in times of famine that the weed is eaten, and then only by the very poor. About 15 hours after ingestion there appears itching of the fingers, quickly followed by swelling and discoloration. This swelling extends up the backs of the hands and outer surface of the forearms. The face also becomes swollen so that the eyelids may be closed, and the nose becomes cyanosed and cold. The swollen parts may ultimately develop blisters and ulcers.

2. Favism.—This, a disease due to ingestion of fresh beans (*Vicia faba*), or exposure to the smell of the blossoms of the plant, appears to be an allergic syndrome, inasmuch as the disease occurs only at the time of the year when the bean is ripening and seems limited to certain families or to individuals with a personal idiosyncrasy. Cases have chiefly been reported from Italy. Shortly after ingestion of the beans,

or even exposure to the flowering plant, an acute, febrile anaemia, with jaundice and haemoglobinuria, sets in. Red cells fall to about 2,000,000 and hb. to 20%. Adults recover quickly, as a rule, but children often die.

3. Lathyrism.—A form of spastic paraplegia due to the eating in times of great scarcity of bread made from the flour of the chick-pea, *Lathyrus sativus*, or other species of vetches. The disease comes on insidiously with pains in the back and weakness of the legs. As the affection develops, the legs are dragged along with great effort, and there is a tendency to fall. Wasting of the leg muscles is common. Reflexes are exaggerated. Incontinence of urine and loss of sexual power are important symptoms. Upper extremities only rarely involved. There is no mental or cardiac involvement. The disease runs a very chronic course but is rarely fatal.

4. Milk Sickness.—Cattle eating richweed (white snakeroot, *Eupatrium ageratoides*) in Eastern United States, or rayless goldenrod in Texas, acquire a serious disease called *trembles*. Man ingesting the milk or butter from a cow so affected suffers from an illness more or less serious according to the amount of toxic material eliminated in the milk. In cattle, the symptoms consist mainly of weakness and uncertain gait, together with clonic muscular contractions which give rise to the descriptive name *trembles*. In man there are observed anorexia, nausea, and vomiting, which prevent the taking of food and water, and soon bring about an acidosis characterized by a high mortality. The later symptoms are subnormal temperature, extremely low blood pressure, presence of diacetic acid in the urine, and of the odor of acetone in the breath and urine. Treatment should be directed toward restoring the acid-base equilibrium.

5. Vomiting Sickness of Jamaica.—This, a disease of the West Indies, is due to eating unripe "ackee," the local name of the fruit of *Blighia sapida*. The fully ripe, opened fruit is harmless and is a valued food. Children, eating the unripe fruit, the arilli of which contain the poison, become suddenly ill in about 2 hours with abdominal pain and vomiting. There is a period of apparent recovery lasting a few hours after which vomiting again begins, and almost coincidently convulsions and coma, ending generally in death.

6. Miscellaneous Sources of Plant Poisoning.—In addition to the diseases described, numerous other instances of poisoning by plants are known, some accidental in origin, and some intentional.

Chestnut, however, states that in the United States there are only about 30 species of plants ever associated with accidental poisoning in man, poisoning with them generally resulting from confusing poisonous plants and harmless ones, as mistaking water hemlock for edible roots. Prussic acid occurs in many valuable foods, and, if not removed, may produce serious results and even death. Bitter cassava, for example, contains a glucoside which in the presence of water sets free HCN; and, in order to avoid poisoning, the tuber must be scraped, grated, squeezed free of its milky juice, and then thoroughly washed. Oxalic acid likewise is contained in variable quantity in many edible plants, as sour grass and rhubarb, and may cause poisoning if ingested in sufficient quantity. Ergotism is another well-known illustration of accidental poisoning from the ingestion of food plants normally wholesome.

In the preparation of arrow poisons, which may be cited as giving rise to examples of intentional plant poisoning, many plant extracts are used, the best-known being that made from the root of the *Acocanthera schimperi* by the Somalis.

R—COMMUNICABLE DISEASES

Common Communicable Diseases

Based on Report of Committee of A. P. H. Association

Cause and source	Incubation period	Mode of transmission	Period of communicability	Laboratory diagnosis	Salient clinical features	Methods of control and remarks
1. *Cerebrospinal Meningitis.* Diplococcus intracellularis meningitidis. Naso-pharyngeal and mouth discharges of person with disease and carriers.	2 to 10 days (commonly 7).	Contact, droplet infection from sneezing or coughing of carriers or cases. Well carriers particularly dangerous to children. Indirect contact by articles freshly soiled by infected persons.	So long as meningococcus recovered from posterior naso-pharynx. "Persistent" carriers (3 months or longer) more common than formerly supposed.	Cultures from nose and pharynx of carrier or spinal fluid of case. Sp. fluid shows increased amount and pressure and turbidity from pus cells. Meningococci may be found in pus cells or extracellular. Leukocytosis. Blood cultures often positive.	Rapid onset with marked headache, stiffness of neck and vomiting. Cutaneous hyperaesthesia. Often Kernig and Brudzinski signs. Fever irregular. Reflexes variable and unequal. Temperature rarely above 102°. Pulse relatively slow. Cases start as blood infection often showing early purpuric rash.	Isolation of case or carriers who have come in close contact with infected individuals until pharyngeal cultures negative 2 to 4 times. Case should be isolated at least one week after fever subsides even with negative cultures. Vaccine immunization doubtful value. Polyvalent antiserum necessitated as it takes time to identify type. Carriers rarely develop disease. Prevent overcrowding and deficient ventilation.
2. *Chicken Pox* (Varicella). Cause unknown. Skin and mucous membrane lesions.	14 to 21 days.	Contact with patient or articles soiled by discharges of lesions.	Until primary scabs have disappeared from skin and mucosal lesions.	Inoculation (Lymph) rabbit's cornea negative for Guarnieri bodies as against variola.	Slight or no backache or vomiting. Back of hands and forearms free from eruption. Eruption chiefly on trunk. Successive crops on same part of body. Soft papules by 2d day becoming unicellular vesicles within few hours.	No immunization. Exclude patient from school and contact with susceptible children. Virus less resistant than that of variola.
3. *Diphtheria.* B. diphtheriae. Throat, nose, vaginal, conjunctival and wound surface discharges.	2 to 5 days. Longer when carrier stage precedes case.	Contact with dischargesfrom case or carrier. Fomites or through milk or milk products.	Until bacilli disappear from discharges or lesions. Healthy carriers may carry infection for months. "Persistent" carriers often show virulent bacilli in tonsillar crypts.	Cultures throat, nose or other affected surfaces on Löffler's blood serum or egg media. B. diphtheriae shows polar granules and virulence for g. p. Intracutaneous injection of $\frac{1}{50}$ M. L. D. toxin shows inflamed area if immunity not present.	Onset more insidious than for scarlatina, follicular tonsillitis or streptococcal angina. Fever less (101° to 102°F.). Pulse tends to show arrhythmia. Albuminuria. Membrane pultaceous, tends to spread and leaves bleeding surface on removal soon reforming. Glandular enlargement angle of jaw. Dyspnoea marked in laryngeal cases. Naso-	Isolate case or carrier until one obtains two negative cultures from nose and throat at least 2 days apart. After lesions disappear bacilli usually disappear in 2 weeks—rarely they persist 2 to 6 months. Carry out Schick tests on contacts and nurses. Exposed susceptibles that cannot be kept

				(Schick test).	pharyngeal palsies may come on (post-diphtheritic).	under daily observation to be promptly immunized by antitoxin. Prophylactic injection of anti-diphtheria serum protects only for 10 days. Immunization with toxin-antitoxin mixture more lasting and probably valuable. Disinfect fomites. Pasteurize milk.
4. *German Measles* (Röthein or rubella). Cause unknown. Oral and possibly nasal secretions.	10 to 21 days.	Direct contact with patient or fresh oral secretions.	Eight days from onset of disease.	None. Large mononuclears stated to be increased at end of disease.	Rose-red macular throat eruption. Rose-pink skin eruption first appears on face. Disappears in 2 or 3 days. Course mild. Presence hybrid rash—morbilliform on face, scarlatinal inner surface thigh, characteristic. Coryza generally slight.	Isolate patient 8 to 10 days. Keep contacts from school 3 weeks. No quarantine. Eruption most marked about mouth and nose. Extends to trunk and extremities by crops in 24 hours. Throat condition and swelling of post. neck glands may simulate scarlatina. Drug and ptomaine eruptions may simulate.
5. *Influenza.* Undetermined. B. influenzae? B. pneumosintes? Filtrable virus?	Short, usually 24 to 72 hours.	Direct contact is popular view, but direct transfer experiments negative. Conjunctival atrium by droplet infection possible. Freshly soiled fomites incriminated.	Undetermined. Probably during first 7 days or even before symptoms.	In absence of secondary infections, a leukopenia.	Abrupt onset. Marked prostration. Muscular soreness, especially ocular and in back groups. More or less conjunctival injection. Pulse slow relative to fever, which shows rapid rise to 102°–104°. Epistaxis and protracted cough common.	Prompt recognition of early symptoms and isolation during acute stage. No proven method of immunization. During epidemics avoid crowds, movies, street cars. Masks recommended for nurses but for populace not proven of definite value. Disinfection of rooms futile. Well recognized clinical types—coryzal, bronchial, nervous and gastrointestinal probably secondary infections invading different parts of body, the unknown virus reducing resistance. Complicating pneumonias and middle-ear disease from various secondary invaders. Cardiac arrythmias and anginal attacks not rare. Protracted nervous and physical depression not uncommon.

R—COMMUNICABLE DISEASES (*Continued*)

Cause and source	Incubation period	Mode of transmission	Period of communicability	Laboratory diagnosis	Salient clinical features	Methods of control and remarks
6. *Measles* (Morbilli). Filterable virus. Oral and nasal secretions.	7 to 14 days (usually 10).	Direct contact with patient or through indirect contact by means of articles freshly soiled with oral or nasal secretions.	While oral and nasal mucous membranes involved. 2 days before and 5 days after rash.	Leukopenia with decrease of lymphocytes before appearance of Koplik spots.	First 3 days coryza with marked fever and malaise. Koplik spots on 1st or 2d day. Characteristic rash about 4th day, first on face. Crescent grouping. Face appears swollen. Conjunctivitis and bronchitis common.	Isolate patient during period of communicability. Quarantine children exposed to infection from school and public gatherings 14 days. Isolate contacts showing 1°F. rise in temperature. No artificial immunization.
7. *Mumps*. Epidemic parotitis. Secretions of mouth and possibly nose. Microorganism not known.	12 to 26 days. Usually 18 days, common maximal is 21 days.	Direct contact with case or articles freshly soiled with secretions of mouth or, possibly, nose.	Unknown but probably until parotid gland returns to normal.	Relative increase in mononuclears of blood.	Pain on opening mouth, chewing or swallowing. Parotid enlargement front and below ear of one or both sides. At times other salivary glands involved. Stomatitis.	Isolate patient for period of presumed infectivity. Quarantine exposed children from school or public gatherings at least 14 days. No immunization. In early diagnosis look for inflammation of Steno's duct. Consider metastatic orchitis.
8. *Poliomyelitis*. Filterable virus. Nose, throat and bowel discharges.	3 to 10 days (commonly 6 days).	Direct contact with case or carrier. Indirectly by articles freshly soiled with virus.	Not known. Apparently not more than 21 days from onset of disease.	Usually slight leukopenia. Occasionally moderate leukocytosis. Sp. fluid clear, moderate lymphocytosis, increased protein and pressure.	Onset may resemble common febrile diseases of childhood or meningitis. Fully developed flaccid paralysis, most often lower extremities (75%) comes on from 1 to 7 days.	Isolation of cases in screened room. Quarantine of exposed children, also of adults who deal with children for 14 days from exposure to case. Disinfect nose, throat and bowel discharges. Healthy carriers supposed to be common.
9. *Scarlet Fever* (Scarlatina). Streptococcus scarlatinae. Discharges nose, throat and ear, and suppurating glands.	2 to 7 days (usually 3 or 4 days).	Direct contact with case or articles soiled by infectious discharges or by contaminated milk.	Three weeks from onset of disease and until all abnormal discharges have ceased and open sores healed.	Nasopharyngeal cultures show haemolytic streptococci. Marked leukocytosis in severe cases. May be some eosinophilia. Examine urine for albumin. Schultz-Charlton reaction. Dick test.	Sudden onset with vomiting, tendency to high fever, tachycardia, angina and enlarged glands at angle of jaw. Boiled-lobster-like eruption by end of 24 hours appearing first on neck and upper chest. Most marked about armpits and region of Scarpa's triangle.	Isolate case 28 days. Carry out Dick test on contacts and nurses. Susceptibles not showing haemolytic streptococci in throat or if showing streptococci but no fever or other symptoms to be actively immunized with scarlet fever toxin. Sus-

					Consider possibility of drug or serum rashes. Look for strawberry tongue. Angina may be membranous and show streptococci in smears. Desquamation in flakes.	ceptibles with haemolytic streptococci in throat, fever or other symptoms, to receive scarlet fever antitoxin (sufficient to neutralize 90,000 S. T. D.). Disinfect all discharges. Pasteurize milk.
10. *Smallpox* (Variola). Cause unknown. Possibly a filterable virus. Lesions of skin and mucous membranes.	8 to 18 days. Sometimes up to 21 days.	Personal contact, also by articles soiled with discharges. Flies may transfer virus.	From first symptoms to disappearance of scabs and crusts.	Reduction of polynuclears with increase of lymphocytes until pustule formation—then leukocytosis from mixed (pyogenic) infection. Immediate vaccination reaction (24 to 48 hours) shows immunity to smallpox. Inoculation tests (Paul's, etc.).	High fever, marked headache, backache, and vomiting for 3 days. Rarely initial scarlatinal or measly rashes on 2d day. Shotty papular rash at hair line and wrists on 4th day with fall of fever. Eruption on trunk and extremities follows facial one in 24 hours. Multilocular vesicles on 6th day. Umbilicated pustules, with rise of fever on 8th day. Same type lesion on same region.	General vaccination in infancy. Revaccination at school entry or in times of unusual prevalence. Hospital isolation in screened wards. Disinfection of all discharges or soiled articles. Segregation of all contacts 16 days or until protected by vaccination.
11. *Whooping cough* (Pertussis). Bordet-Gengou bacillus. Bronchial or laryngeal secretions. Sometimes dogs and cats.	Within 14 days.	Direct contact with cases or articles freshly soiled by discharges.	Particularly contagious in early stages before characteristic whoop. Communicability probably lasts 2 weeks after whoop.	Primary leukocytosis, then lymphocytosis, then eosinophilia. Examine smear from sputum of early stages and culture same.	Onset with coryza or bronchitis for 3 to 14 days before development of characteristic "whoop."	Isolate child 2 weeks after "whoop." Disinfect discharges. Cats and dogs susceptible and may transmit disease. Vaccination possibly of prophylactic value.

INDEX OF ESSENTIAL LABORATORY PROCEDURES

Frequently grave issues turn upon a laboratory report. A false positive may ruin the happiness of a family or entail great economic loss in needless quarantine or destruction of wrongly suspected materials; a false negative on the other hand may lead to disaster in a social group or expose a community to the invasion of disease. Consequently persons responsible for management of a laboratory should be ever on the alert to exclude error from their reports, and persons whose decisions in the conduct of affairs are dependent in whole or in part upon laboratory reports should be qualified to appraise them correctly.

Abscess.—Take up material, observing asepsis, with pipette, hypodermic syringe or loop. Plate out on agar and blood agar. Stain smear by Gram and Romanowsky and, when indicated, acid-fast method. Usually leukocytosis. If large and putrefying, indicanuria. (See following: also "Empyema," "Furunculosis.")

Abscess, liver.—Leukocytosis 12,000–20,000. Normal polymorphonuclear percentage. Large mononuclears of blood increased. Pus obtained by aspiration is like anchovy sauce. Amoebae may not be found for several days after drainage is established. Contents often sterile, pus cells rare. Roentgenogram. (See "Abscess," "Liver Disorders.")

Abscess, lung.—Large amounts of pus and fragments of lung tissue, and elastic fibers, may be found in sputum. Lung puncture may be considered. (See "Abscess.")

Acidosis.—The best index is the plasma CO_2 capacity (p. 712). Examine urine for urea, which is usually low, acetone bodies and NH_3 quotient (p. 712). (See also p. 708, et. seq.)

Actinomycosis.—Look for large yellow granules in pus. Press out granules between slides and stain by Gram's method. The central mycelium is Gram-positive while peripheral "clubs" are Gram-negative. Anaerobic culture. See page 206.

Acute yellow atrophy of liver.—Urine usually shows low urea, high NH_3 quotient, and leucin, tyrosin and bile. Bear in mind the possibility of acidosis.

Addison's disease.—Eliminate jaundice by examination of blood serum and urine for bile. Urobilinuria is uncommon. Blood chemistry shows no hypoglycaemia after injection of epinephrin. Lymphocytosis.

Amoebiasis.—See page 398.

Anaphylaxis.—See "Hypersensitiveness."

Ancylostomiasis.—Characteristic ova in faeces. Eosinophilia. See page 488.

Anaemia, aplastic.—Severe and increasing anaemia which runs a rapidly fatal course. There are no pathological red cells found in the blood. There is apt to be a leukopenia, with reduction of granular cells derived from bone marrow. Polymorphonuclears are progressively reduced. Blood platelets decreased. See page 357.

Anaemia, chlorotic.—Achromia. Color index very low. Volume index normal or low. Macrocytes and megaloblasts always absent.

Anaemia, pernicious.—Low red count, often 2,000,000 or less. High color and volume indices. Anisocytosis, especially the presence of macrocytes. There is

poikilocytosis. Megaloblasts usually present. Myeloblasts and myelocytes may be present. Remissions may change blood picture toward normal. Blood chemistry, especially for cholesterol and bilirubin content (Table, p. 696). Urobilinuria is common. See page 355. Free HCl (gastric) absent.

Anaemia, posthaemorrhagic, acute.—Low color index. Volume index normal or low. Normoblasts usually found third day after haemorrhage. Blood platelets increased; coagulation time decreased. Usually polymorphonuclear leukocytosis with an occasional myelocyte. Urobilinuria is usual if haemorrhage is internal.

Anaemia, posthaemorrhagic, chronic (as in gastric cancer, etc.).—It may more nearly approach color index of pernicious anaemia. Volume index normal or low. Moderate leukocytosis, few poikilocytes, with many microcytes.

Anaemia, sickle cell.—See page 357.

Angina, streptococcal.—Make culture on Loeffler's serum—the water of condensation is excellent for demonstrating chain formation. Culture on blood agar plate to demonstrate haemolysis or the production of a green-colored colony (*S. viridans;* pneumococci). Stain smears from throat by Grams' method. Organism often appears in pairs or short chains. Consider phagocytosis in the smear.

Angina, Vincent's.—Stain smears by dilute (1 to 10) carbol fuchsin. Vincent's spirilla and fusiform bacilli are found in large numbers. (A few may be found from throats apparently normal.) It is well to culture on Loeffler's serum as diphtheria is at times associated with Vincent's angina. Lymphocytosis. See page 585.

Anthrax.—Large Gram-positive bacilli may be found in direct smear from malignant pustule. Hanging drop shows organisms to be nonmotile. Culture on agar. Inoculate a guinea pig or mouse subcutaneously—death results in 48 hours. At the site of injection there is an oedema and a gelatinous exudate. The blood of the animal is dark and contains large numbers of bacilli. Examine sputum in Woolsorter's disease. See page 112.

Appendicitis.—Polymorphonuclear leukocytosis.

Arteriosclerosis.—Blood chemistry to differentiate essential hypertonia (p. 697) and determine kidney function (q. v.).

Arthritis.—Blood uric acid to differentiate gout (see page 697).

Ascaris infection.—Examine faeces for characteristic ova. Diagnosis often made by worms in stool or in vomitus.

Ascites.—Determine specific gravity, albumin and cell content, etc. In case of exudate make culture. See page 636. Test for ferments to differentiate ruptured pancreatic cyst. (See Oedema.)

Bacteriaemia.—White count often shows polymorphonuclear leukocytosis, except in those conditions in which leukopenia is an accompanying feature. Make blood culture. See page 620.

Bacteriuria.—Examine sediment in hanging drop and stained smear. Collect catheter specimen under sterile precautions and plate out on agar and blood agar. In males such precautions may not be necessary. See page 609.

Balantidium infection.—Look for large motile ciliates in faeces.

Blackwater fever.—Haemoglobinuria. Pink foam to urine. Malarial parasites may be found in blood smears in some cases.

Botulism.—Inject an infusion of the suspected ham, sausage or vegetable, into a guinea pig. In positive cases paralysis of eye muscles and later death of the guinea pig result from cardiac and respiratory failure. Cultures may be made on glucose

agar, kept at room temperature, in a dark place and under anaerobic conditions. See page 118.

Carbon-monoxide poisoning.—Test blood for CO.

Cerebrospinal fever.—Leukocytosis. Make blood culture. Lumbar puncture. Abundant polymorphonuclears and eosinophiles usually found in spinal fluid. Gram-negative intra and extracellular diplococci. Culture fluid on blood agar. There is a globulin increase. Blood culture may demonstrate the presence of organisms before they can be found in spinal fluid. Consider carriers (posterior nasopharynx). See page 103.

Cestode infections.—Examine faeces for ova, which are not always readily found. If a segment is obtained, press between two glass slides and examine the branchings of the uterus. See page 464.

Chancroid—Ducrey's bacillus.—Short cocco-bacilli occurring in chains—Gram-negative. Consider culturing. Material for culture should be obtained before ulceration occurs. Dark-field illuminator will assist differentiation from chancre.

Children.—(See "Pediatrics.")

Cholecystitis.—May be moderate leukocytosis. Duodenal fluid obtained by duodenal tube may show bile-stained pus cells. The presence of bile is an indication that the bile ducts are patent.

Cholelithiasis.—Blood chemistry. (See Table, p. 696.) Test calculi (p. 747).

Cholera.—Make a straight smear with epithelial flakes from rice-water material; dry, fix by heat; stain with dilute (1–10) carbol fuchsin. Spirilla give the appearance of fish swimming parallel to one another. Hanging drop shows extreme motility of spirilla. Make agglutination test, using serum from convalescent patient or immunized rabbit. Normal serum control does not agglutinate higher than 1–20. See page 189.

Chyluria.—Centrifuge urine and examine for filarial embryos. Examine blood at night for filarial embryos (not always found). Many highly refractile fat globules in urine—soluble in ether.

Coma.—Blood chemistry to determine cause. (See p. 697.) Examine urine.

Conjunctivitis.—Stain smear by Gram's method and dilute carbol fuchsin. Culture secretion on blood agar and plain agar. See pages 579–581.

Cyst.—Settle any possible question of connection with the urinary passages by examination for urea.

Cystitis.—Gram's stain of portion of urinary sediment obtained by centrifuging. Look for phagocytized organisms. Plate out a portion of urine on agar and another on blood agar. See page 599. (See Table, p. 610.)

Dengue fever.—Leukopenia and reduction in percentage of polymorphonuclears.

Diabetes insipidus.—Differentiate diabetes mellitus (*q.v.*), and nephritis (*q.v.*).

Diabetes mellitus.—Blood chemistry, especially for sugar, cholesterol, and nitrogenous compounds. (See Table, p. 696.) Examine urine especially for sugar, acetone bodies and NH_3 quotient. Differentiate renal diabetes (p. 697). Bear in mind kidney function and acidosis. Blood sugar is index of efficacy of treatment. (See "Glycosuria.")

Diphyllobothrium infection.—Operculated ova in faeces. If segments are obtained, press one between two glass slides and observe the characteristic rosette-shaped uterus. Blood picture of the pernicious anemia type is sometimes seen.

Diphtheria.—Culture on Loeffler's serum or whole egg medium for 8 hours or more. Stain by Ponder's, Gram's, and Loeffler's methods. Look for parallelism and polar granules. Make Schick test on contacts. Inject guinea pig with culture as test for virulence. See page 141.

Dracunculus infection.—Bathe blister or ulcer with a few cubic centimeters of water. Examine fluid excreted from worm for striated embryos.

Duodenal ulcer.—Analysis of stomach and duodenal contents. (See pp. 622, 746.) Examine these, as well as vomitus and faeces, especially for blood.

Dysentery, amoebic.—Examine mucus from stools for amoebae actively putting forth finger-like pseudopodia. Pathogenic amoebae are apt to contain red cells. Mucus mixed with Gram's iodine solution may show one- to four-nucleated cysts. Smear of faeces shows granular detritus—no pus cells. Culture for amoeba. Large mononuclears in blood increased. See page 392.

Dysentery, bacillary.—Sanguinolent mucus shows abundant polymorphonuclears and many phagocytic endothelial cells. Emulsify a loopful of mucus in sterile bouillon and plate out on Teague or Endo medium. See page 176.

Echinococcus disease.—Complement fixation tests. Examine aspirated fluid from cyst for hooklets. Eosinophilia. See page 472.

Empyema.—Gram's stain of smear from pus. Culture material on blood agar and plain agar plates. Polymorphonuclear leukocytosis in blood. See page 636.

Encephalitis lethargica.—Inoculate rabbits with nasopharyngeal material or spinal fluid. Culture spinal fluid.

Endocarditis.—Blood culture. Plates probably will show *S. viridans.* Moderate polymorphonuclear leukocytosis.

Endocrine disorders.—Blood chemistry. (See Table, p. 696.) (See also "Pancreatic Disorders," "Pituitary Disorders," "Thyroid Disorders" and "Addison's Disease.")

Enteritis, tuberculous.—Acid-fast stain on smear from faeces. Decolorize over night in acid alcohol. There are usually present in faeces acid-fast spore-like bodies which should not be mistaken for tubercle bacilli. Tubercle bacilli may be present in faeces when there is no involvement of intestine. Children, especially, are apt to swallow sputum.

Eosinophilia.—Look for ova of intestinal parasites in faeces. Consider trichinosis, hydatid disease, asthma, filariasis, various skin diseases and diseases of bones.

Favus.—Place hair or portion of favus cup on a slide, drop on 10% sod. hydrate solution, cover by cover glass and examine by $\frac{2}{3}$ and $\frac{1}{6}$ lenses for mycelium and spores which are very irregular.

Filariasis.—Collect a drop of blood on a slide and place cover glass on drop. Seal with vaseline. Examine with $\frac{2}{3}$ lens for embryos. Stain blood films by Giemsa's stain. Blood should be examined both night and day. Eosinophilia often noted. Embryos often absent in peripheral blood in cases of elephantiasis, lymph scrotum, etc., but puncture of engorged lymphatics may show embryos. For Guinea-worm disease see Dracunculus infection. See page 495.

Flagellates.—Examine faeces emulsified in salt solution, with $\frac{1}{6}$ lens for actively moving flagellates. Emulsify another portion in Gram's solution to study flagella. Stain a smear with Giemsa's stain for encysted forms, which may be confused with *Blastocystis.* Culture. See page 50.

Fungi.—Examine scrapings from skin, in 10% sodium hydrate solution.

Furunculosis.—Determine blood sugar. (See "Abscess.")

Gas gangrene.—Make hanging drop and stain smears from exudate by Gram's method. *B. perfringens* (*B. welchii*) is Gram-positive and non-motile. Anaerobic culture on litmus milk and glucose agar stabs. Milk and agar disrupted by incubation over night. See pages 124–127.

Gastric cancer.—Blood may show an anaemia approaching the type of pernicious anaemia. Examine stomach contents. (See p. 622.) Examine faeces for blood.

General paresis.—Lumbar puncture. Spinal fluid shows increased cell count, positive globulin tests, paretic curve with colloidal gold, and positive Wassermann and Kahn test. Blood shows positive Wassermann and Kahn test.

Glanders.—Smears from pus show Gram-negative bacilli. Make cultures on acid glycerin agar and potato. Non-motile organisms showing parallelism. Intraperitoneal inoculation of a male guinea pig causes marked swelling of testicles in 2 to 7 days. Complement fixation tests are of service.

Glycosuria.—Examine urine for sugar. Blood chemistry, especially for sugar. Sugar tolerance test. Consider renal diabetes (p. 697).

Gonococcus infection.—Gram's stain of smear from pus obtained from urethra or eye—shows Gram-negative diplococci. Culture. See page 101.

Gout.—Blood chemistry. (See Table, p. 696.) Uric acid in urine increases before and during an acute attack.

Haematuria.—Centrifuge specimen. Stain sediment by Gram's stain and Ziehl-Neelsen. Culture on blood agar and Petroff's medium. Inoculate a guinea pig.

Haemoglobinuria.—Centrifuge specimen and examine sediment to determine absence of red cells. Shadow cells may be found with much débris.

Hodgkin's disease.—Blood count shows normal to moderate increase in white cells with increased (10%–15%) percentage of transitionals. Blood platelets increased. Best method of diagnosis is examination of excised gland. See page 365.

Hypersensitiveness.—To determine if a patient is sensitized to a serum which is about to be administered to him, inject intradermally 0.2 cc. of a 1–10 dilution of the serum. If he is sensitized an urticarial wheal will develop in about 5 minutes and begin to fade in an hour. See page 294. Sensitization to quinine is shown by the production of a wheal shortly after 10% solution of quinine bimuriate is applied to a scratch.

Infants.—(See "Pediatrics.")

Influenza.—Leukopenia. Sputum often shows small Gram-negative bacilli which tend to group about cocci. Culture sputum on blood agar to note Pfeiffer's bacilli, streptococci, etc. See pages 157, 658.

Intestinal disorders.—Analysis of stomach and duodenal contents. Complete examination of faeces, especially for blood. Indicanuria accompanies stasis and putrefaction. Blood chemistry may be of aid. (See Table, p. 696.)

Intestinal parasites.—Examine faeces for ova.

Jaundice.—Examine duodenal contents, especially with a cholagogue. Examine urine, faeces and blood serum for bile pigments and diastase (pancreatitis). Bear in mind acute yellow atrophy of liver and Addison's disease.

Jaundice, haemolytic.—Increased fragility of red cells. Presence of reticulated cells. See page 367. Van den Bergh reaction. See page 741.

Jaundice, infectious.—Leukocytosis. Inject guinea pig with blood of patient, spirochaetes being found in liver emulsion at autopsy of animal.

Kala-azar.—Marked leukopenia with polymorphonuclear decrease. Blood culture on N. N. N. medium. Smears made from liver or spleen juice and stained by Wright's stain often show organisms, but spleen puncture is a dangerous procedure. Cultures should be kept at room temperature for 2 to 3 weeks for development of parasite. Smears from peripheral blood may show organisms in polymorphonuclears or large mononuclears after prolonged search. Large mononuclears of blood increased. See page 413.

Kidney disorders.—Examine urine. Blood chemistry. Consider kidney function. If there is pus in urine, examine especially for *B. tuberculosis.* (See "Nephritis," "Nephrolithiasis," "Pyelitis" and Table, p. 696.)

Kidney function.—Estimate by use of more than one test. (See p. 732, *et seq.*)

Leprosy.—Make smears from nose and from suspected lesions. It is well to scrape lesions until serum appears. Stain for acid-fast organisms, which are often numerous and may give the appearance of a bundle of cigars. Nonpathogenic for guinea pig. See page 137.

Leukaemia, lymphatic.—Red cells usually reduced about one-half; color index below normal; normoblasts rarely found; myelocytes usually absent but may amount to 5% of leukocytes; small lymphocytes predominate (75% to 98%). In acute lymphatic leukaemia the white count may be normal early but soon increases. In it lymphoblasts and Rieder cells are diagnostic. White counts may range from normal to 150,000 in acute; in chronic does not exceed 125,000. See page 363. Uric acid in blood often markedly increased. Urine may show Bence-Jones' protein.

Leukaemia, splenomyelogenous.—White counts average 200,000 to 500,000 with a large proportion of myelocytes. Urine may show Bence-Jones' protein.

Liver disorders.—Urobilinuria accompanies impaired function. Examine duodenal contents. (See "Abscess of Liver," "Acute Yellow Atrophy of Liver" and "Cholelithiasis.") See liver function tests, page 738.

Madura foot.—Discharge contains fish roe granules, which show mycelium and peripheral club-like structures.

Malaria.—To examine unstained blood place a drop on slide and cover by cover glass. Seal edges by vaseline. Fresh preparations are not as valuable as the stained smears. To decide that an object is a malarial parasite in stained smears one should always have at least two of three things: (1) Chromatin; (2) bluish or greenish cytoplasm, (3) pigment. Crescents are diagnostic for maligant tertian; equatorial banding for quartan. Marked irregularity of outline of parasite and the presence of Schüffner's dots (reddish) in cytoplasm of red cells speak for benign tertian. May show positive Wassermann during paroxysm. See page 421.

Measles.—Diazo-reaction in urine usually precedes eruption, and is not found in German measles.

Myeloma, multiple.—Bence-Jones' protein quite constantly in urine.

Nephritis.—Examine urine, especially for albumin, casts, blood and pus. Blood chemistry, especially for nitrogen and chloride retention. (See Table, p. 696.) Bear in mind kidney function, uraemia and acidosis, as well as the peculiarities of acute nephritis. (See p. 697.) A steady decrease in nitrogenous compounds in

urine gives a bad prognosis; when sudden, it usually foretells uraemia. It is well to guide diet, etc., by blood chemistry and Mosenthal's method. (See "Kidney Disorders," pp. 722–738 and Table, p. 696.)

Nephrolithiasis.—Examine urine especially for "sand," clumped calcium oxalate crystals and blood.

Oedema.—Urine usually shows low NaCl except during periods of elimination. Blood chemistry, especially for NaCl, and as an aid in determining cause. Ascertain bearing of kidney function upon condition. (See Table, p. 696.)

Ophthalmia neonatorum.—See Gonococcus infections.

Oriental sore.—Stain smear made from scrapings of base of ulceration by Wright's stain for Leishman bodies.

Oroya fever.—Acute, rapidly developing anaemia resembling that of pernicious anaemia. Rod-like organism, *Bartonella bacilliformis*, found in red cells and blood culture.

Otitis media.—Polymorphonuclear leukocytosis in blood. Make culture from discharge on blood agar and plain agar. If mastoiditis consider blood culture.

Pancreatic disorders.—Examine duodenal contents, especially for enzymes. (See p. 622, *et. seq.*) Blood chemistry, especially for sugar and diastase. (See Table, p. 696.) Examine faeces by Schmidt's test, and also particularly for undigested muscle fibers, and abnormal amounts of neutral fat or fatty acids.

Paragonimiasis.—Examine fresh sputum for light-yellow, operculated ova, averaging 90 × 65μ.

Paratyphoid fever.—Examine as for typhoid fever. Use glucose fermentation tube. Paratyphoids give gas. See page 181.

Pediatrics.—Bear acidosis constantly in mind. Examine urine for acetone bodies.

Pertussis.—Leukocyte count 15,000 to 50,000, lymphocytes increased. Gram's stain of sputum may show small oval, Gram-negative, bipolar bacilli. Cultures made on blood agar or ascitic agar. The organisms are found in sputum at the beginning of the disease.

Piedra.—Examine hairs for small gritty masses which consist of spores arranged like mosaics about hairs.

Pituitary disorders.—In hyperpituitarism (acromegaly) there may be polyuria and glycosuria connected with hyperglycaemia. Calcium retention supposed to exist. In hypopituitarism (dystrophia adiposo-genitalis—Frölich syndrome) we may have low blood pressure and subnormal temperature. Important is the greatly increased sugar tolerance so that patient may require the ingestion of as much as 500 grams glucose to produce glycosuria. This sugar tolerance may be reduced by injection of extract of the posterior lobe. See "Endocrine disorders."

Plague, bubonic.—Obtain material by gland puncture, smear on slide and stain by methylene blue. Look for oval, bipolar-staining organism. Inoculate guinea pig cutaneously or subcutaneously. See page 166.

Plague, pneumonic.—Stain blood-tinged sputum. Inoculate animal.

Plague, septicaemic.—Withdraw from a vein 5 to 10 cc. blood for culture in bouillon. Prepare heavy blood smears—organism can at times be found in direct smears. Leukocyte count 12,000 to 15,000.

Pleural fluid.—Collect small portion in citrated salt solution; centrifuge, treat sediment with 1% formalin, centrifuge again and stain sediment by Gram's stain,

Wright's stain and Ziehl-Neelsen. Collect another portion of fluid for culture on blood agar and inoculation of a guinea pig. In tuberculosis the differential count of cells in fluid usually shows a lymphocytosis. See page 636.

Plumbism.—Moderate anaemia, 2,500,000 or more red cells. Red cells show large bluish granules—punctate basophilia. Nucleated red cells are often found. Urobilinuria may be present. Blood chemistry. (See Table, p. 696.)

Pneumonia, broncho-.—Culture sputum on blood agar for haemolytic streptococcus, Pfeiffer bacillus, etc. Make blood cultures. Consider possibility of streptococcal empyema.

Pneumonia, croupous (pneumococcus).—Culture sputum on Avery's medium. Determine type of pneumococcus by specific agglutinating serum. Blood count shows polymorphonuclear leukocytosis. Urine chlorides may be nil. Bear possibility of acidosis in mind.

Poliomyelitis.—Spinal fluid shows lymphocytosis and reduces Fehling's solution. Noguchi and Flexner have cultivated a "globoid" organism which is able to pass through a Berkefeld filter. They cultivated the organism in media prepared as for culturing *Treponema.* Secretion from nasal mucosa contains the organism. Monkeys have been successfully inoculated.

Pregnancy.—Blood chemistry (see Table, p. 696), high uric acid usually meaning toxaemia. Examine urine, toxaemia usually being accompanied by acetone bodies and increased NH_3-quotient. Acidosis and impairment of kidney function are usually mild when occurring but should be watched for.

Prostatic hypertrophy.—Blood chemistry (see Table, p. 696), and examine urine. Bear kidney function in mind.

Pyelitis.—Culture urine obtained by catheterization of ureters on blood agar. Stain smears from sediment of centrifuged specimen. Look for phagocytized bacteria.

Rabies.—Keep dog alive, which has bitten patient, to observe symptoms. If dog has been killed make smears from cornu Ammonis and stain by Giemsa's stain or by basic fuchsin and methylene blue. Negri bodies. See page 649.

Relapsing fever.—Examine blood smears stained by Wright's stain for spirochaetes. The dark-field illuminator and India ink methods are also of service. Spirochaetes may be absent from peripheral blood during apyretic period.

Scabies.—With the aid of a hand lens seek a part of infected skin showing black line which marks the tunnel for the parasite. The female can be found at the end of tunnel and removed by a needle. If parasite is not obtained diagnosis can be made by finding ova in scrapings from skin. See page 511.

Scarlet fever.—Leukocytosis 12,000–15,000. Cultures from throat show haemolytic streptococci. Schultz-Charlton reaction. Make Dick test on contacts. Watch urine for evidence of nephritis.

Schistosomiasis.—Examine urine, faeces or rectal discharges for ova.

Screw-worm disease.—Examine breathing slits on posterior stigmata of larvae found in auditory canal.

Septicaemia.—Make blood culture. Blood usually shows polymorphonuclear leukocytosis.

Smallpox.—Initial leukopenia followed by leukocytosis, 12,000–15,000 in pustular stage. Large mononuclears increased. Inoculation tests (Paul's, etc.).

Sporotrichosis.—Culture on potato or agar for 8 days or more. Direct smears do not show organisms.

Sprue.—Tongue scrapings and stools usually show *Monilia.* Stools show 25 to 40% fat. Blood calcium diminished. Hypochlorhydria. May have anaemia of pernicious type.

Stomach disorders.—Analysis of stomach contents. (See p. 622.) Blood chemistry and urine examination to eliminate nephritis.

Surgery.—Bear in mind especially acidosis, liver and kidney function. (See pp. 708, 732, and 738.

Syphilis.—Initial sore. Examine serum by dark-field illuminator or stain smears by the Warthin-Starry silver-agar method or Tribondeau's method. Consider gland puncture and tissue transfer. Complement fixation and precipitation test of blood and if required of spinal fluid. Cell count spinal fluid; globulin test; colloidal gold test. Large mononuclears of blood increased. See pages 445–447.

Thrombocytopenia.—Blood platelets markedly diminished. Bleeding time prolonged. Clotting time usually normal but clot fails to retract. See page 359.

Thrush.—Make scrapings from lesions and examine in 10% sod. hydrate solution. The organism, *Monilia albicans,* may be cultivated on Sabouraud's media. It slowly liquefies gelatin and blood serum and, after acidifying, clots milk. In cultures there are budding yeast-like forms and mycelial threads.

Thyroid disorders.—The clinical picture of marked thyroid disturbances is so striking as to suffice in ordinary diagnosis. For more scientific study of a case and particularly in those cases where the clinical manifestations are not marked we should consider certain laboratory investigations. The three prominent methods for such study are (1) blood chemistry, (2) epinephrin test and (3) basal metabolism estimation.

1. *Blood chemistry.*—Sugar, urea and diastase are usually increased, especially at noon, in hyperthyroidism, except during a fast, but do not necessarily parallel basal metabolism. Hypothyroidism usually shows low values for sugar, urea and chlorides. (See Table, p. 696.) The sugar tolerance test (p. 700) gives a blood sugar of about 230 in one hour and it may require two or more hours to reach normal. A positive result serves to corroborate a diagnosis of hyperthyroidism; a negative is 90% accurate as an indication of the absence of the affection. The following epinephrin test affects the blood sugar as does the sugar tolerance test.

2. *Epinephrin test.*—In the test as recommended by Goetsch the patient after resting in bed for one hour receives a hypodermic of 0.5 cc. of a 1 to 1000 epinephrin solution in the deltoid muscle. Prior to giving the epinephrin, notes are made as to blood pressure, pulse rate, respiratory rate and existence of tremor. Following the injection we take the blood pressure, pulse and respiratory rate at intervals of two minutes for ten minutes. Then at intervals of five minutes for one hour, and then every ten minutes for one-half hour. In hyperthyroidism, when thyrotoxicosis is present, we get increase in systolic blood pressure of more than ten millimeters with a corresponding increase in pulse rate. The reaction shows itself in about 15 minutes and is over in a little more than half an hour. The pulse pressure is usually increased. In addition a positive reaction should show symptoms of flushing, increased tremor, etc. The value of the test is descreased by the fact that positive reactions have been obtained in 14% of normal individuals, in about 50% of cases of effort syndrome and in about 50% of individuals convalescing from acute infections.

3. *Basal metabolism.* In the study of cases in which abnormal thyroid functioning is suspected the most accurate method of investigation is by estimating the variation of the patient's basal metabolism above or below the normal average. The basal metabolic rate is the caloric value of the heat which an individual produces while resting in bed and at least fifteen hours after the ingestion of food, expressed in terms of calories per hour per square meter of body surface. It varies with different ages and is proportionate to the body surface. Normally this metabolic rate should not vary as much as 15% above or below figures accepted as normal for a given surface area. The use of the respiratory calorimeter is the most accurate method for the determination of heat production, but it has been shown that the oxygen consumption during short periods gives data from which the heat production may be accurately determined (indirect calorimetry). In the Benedict portable respiration apparatus, the patient breathes into and out of a confined volume of oxygen, circulating through a series of purifiers which remove the carbon dioxide. The volume of oxygen consumed is ascertained by noting the decrease in the total volume confined in the machine, and must be corrected to standard conditions (760 mm. mercury, 0°C.) for accurate determination. The heat production resulting from the absorption of one liter of oxygen is relatively constant if the patient has been on an average diet. The Benedict apparatus can also be employed for determining carbon dioxide excretion and thereby giving data for the determination of the respiratory quotient (ratio between O_2 absorbed and CO_2 given off). The determination of the heat produced from the excretion of carbon dioxide is less accurate though easier of determination. In marked cases of hyperthyroidism, the basal metabolism ranges 75% above the normal figures, between 50 and 75% for severe cases, and less than 50% for mild cases. In hypothyroidism, the figures are usually 20 to 40% below the normal averages. Severe cardiac and renal conditions, as well as leukaemias, also give increased rates. It must be remembered that patients with high fever give rates 30 to 40% or more above normal.

If the patient has been on a normal diet, the respiratory quotient will be approximately 0.82, and at this figure each liter of oxygen absorbed has a caloric value of 4.825. The rate is usually reported in percentage above or below accepted normal standards. The body surface area is determined from the height and weight according to the formula or the chart devised by DuBois. The calculation is expressed in the following formula:

$$\frac{\text{L of } O_2 \times (\text{factor cor. to 760 mm. and 0°C.}) \times \dfrac{1 \text{ hour}}{\text{time of test}} \times 4.825}{\text{Sq. M. of body surface}} = \text{B.M.R}$$

NORMAL STANDARDS

AGE	MALES	FEMALES
14–16	46.0	43.0
16–18	43.0	40.0
18–20	41.0	38.0
20–30	39.5	37.0
30–40	39.5	36.5
40–50	38.5	36.0
50–60	37.5	35.0
60–70	36.5	34.0
70–80	35.5	33.0

Example: Patient: Male 29 yrs., 5 ft., 8¾ in., 147½ lb.
Body surface, 1.8 sq. M.
Time of test: 5 min., 7 sec.
Bar. 30.11 in.
Temp. of O_2 at start, 23.5°C. Factor 0.926.
Vol. O_2, 7200 cc.
Temp. of O_2 at end, 24.0°C. Factor 0.925.
Vol. O_2, 4900 cc.
$7200 \times 0.926 = 6667.2$ cc.
$4900 \times 0.925 = \underline{4532.5}$ c.c.
2134.7 cc., or 2.1347 liters of O_2 at 760 mm. and 0°C

$$\frac{2.1347 \times \frac{3600}{307} \times 4.825}{1.8} = 67.1 \text{ Cal.}$$

$\underline{39.5}$ Cal. (Normal).
27.6 Cal. per hour per sq. M. body surface above normal.

$x : 100 = 27.6 : 39.5$
$x = 69.87\%$, or B.M.R. is approximately 70% above normal, or $+ 70\%$

It is better to take three successive readings similar to above a few minutes apart and determine the average. If the three readings are not approximately the same, it suggests an error of technique.

At the U. S. Naval Medical School we now use the portable Roth-Benedict apparatus and as an aid in simplifying calculation we find the Roth metabolimetric chart very useful.

Reed's formula.—A method for calculation of the basal metabolic rate from the basal pulse rate and basal pulse pressure in cases in which gross cardiovascular lesions are absent.

B.M.R. $= 0.683$ (P.R. $+ 0.9$ P.P.) $- 71.5$
Example:—Pulse rate 100. Pulse pressure 70 (150–80).
B.M.R. $= 0.683 (100 + 0.9 \times 70) - 71.5$
$= +39.8$

Reed found an error of only 10% in 60% of cases, and of 20% in 91% of cases. This error may be either a plus or minus one.

Our experience, in a limited number of cases, showed a fairly close agreement in the the B.M.R. obtained by Reed's formula and the portable Benedict apparatus.

Transfusion—blood grouping tests. It is not sufficient to get men in same group for transfusion. There should be direct matching—the cells of donor against the serum of recipient and cells of recipient against the serum of donor. Wassermann or Kahn tests should be carried out and examination for malaria in blood of donor. Dried Group II and Group III serum will keep for some weeks in refrigerator. See page 329.

Trichinosis.—Eosinophilia. A small piece of deltoid muscle may be excised and examined. Centrifuge blood treated with acetic acid for embryos. Faeces may show adult worms during diarrhoeal stage. See page 479.

Trypanosomiasis.—Collect 10 to 20 cc. blood in citrated salt solution. Examine leukocyte layer for trypanosomes. Make smears from gland juice. In advanced cases trypanosomes are found in spinal fluid. Inoculate guinea pigs intraperitoneally with blood or gland juice. See page 405.

Tuberculosis.—Acid-fast stain of smears from sputum and faeces, or sediment from urine. Inject guinea pig subcutaneously with material. Culture on Petroff's medium. Blood cultures on Petroff's medium may be positive in miliary tuberculosis. See page 135. Excluding febrile complications, a persistent positive Diazo reaction in the urine gives a grave prognosis, Todd stating that death usually follows in six months.

Typhoid fever.—Leukopenia with relative increase in lymphocytes. Blood culture during first week. Agglutination test after 7 to 10 days. Culture urine and faeces on Endo or Teague medium. Eosinophiles are decreased or absent. See page 176.

Manson-Bahr regards the *Marris atropine test* as of the utmost value in the diagnosis of the enteric group fevers. In this test one gives a hypodermic injection of grain $\frac{1}{50}$ of atropine sulphate. Should the case be typhoid or paratyphoid the pulse rate is practically uninfluenced during the period from 25 to 50 minutes after the injection. In other infections or in normal individuals, the pulse rate drops at first but after 10 or 15 minutes rises to exceed the pulse rate before the injection by 30 or 40 beats during the period of 25 to 50 minutes following the injection.

Typhus fever.—Increased white count with normal percentage of polymorphonuclears. See Weil-Felix agglutination, page 185.

Undulant fever.—Blood culture should be made in the afternoon and at the height of the fever. Incubate for at least four days. Urine may also be cultured. Agglutination tests may be positive as early as the fifth day.

Yellow fever.—At the onset hyperleukocytosis which in a few days drops to normal or below (leukopenia). The polymorphonuclears are increased in percentage. Albuminuria sets in by 1st or 2d day to increase later on with granular and epithelial casts by 2d or 3d day. Diminution in amount of urine or suppression in fatal cases. Urine and blood serum show bile pigments by 2d or 3d day. Injection of blood of patient (in first three days) intraperitoneally into guinea pigs infects about 10% of animals, which show *Leptospira icteroides* in liver and blood. Cultures may be made from blood of patient or guinea pigs. Examine with dark-field illuminator.

Note.—While recognizing the value of the Roentgen ray as an aid in diagnosis, it has not been found possible to discuss its uses in the preceding index to laboratory procedures. However, it is well to remember that it may be of assistance in the following conditions: subdiaphragmatic abscess, abscess of lung and liver, dental infections, actinomycosis of bone, arthritis, cholecystitis, cholelithiasis, echinococcus cysts of lung and bone, empyema, gastric cancer and ulcer, gout, kidney disorders, leprosy, madura foot, multiple myelomata, mastoid infection, aneurysms and pericarditis, pleural effusions, unresolved pneumonia, prostatic hypertrophy, stomach disorders, syphilis, surgical conditions, substernal thyroid, persistent thymus, tuberculosis, typhoid in bone, duodenal ulcer, tumors and newgrowths.

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UNITS IN COMMON USE IN LABORATORIES

Cubic Meter.—Unit of space for the number of organisms in air. It contains 1000 liters. It is equal to 1.308 cubic yards or 35.316 cubic feet. One thousand cubic feet, the unit of space in disinfection, is equal to 28.3+ cubic meters. One cubic decimeter is one liter and equals 0.908 quarts dry measure or 1.0567 quarts liquid measure.

Liter.—Unit of space for normal volumetric solutions. It contains 1000 cubic centimeters. It is equal to 1.0567 quarts or 33.8+ ounces. A liter of distilled water weighs 1 kilogram. One U. S. gallon is equal to 3785 cc. and one imperial gallon to 4543 cc. One fluid ounce equals 29.57 cc.

Cubic Centimeter.—Unit of space for organisms in water, milk, vaccines, etc., 1 cc. = 0.27 fl. dr. There are, approximately, 20 drops in 1 cc. of water, provided the capillary pipette has a bore of about 1 mm. and is held horizontally. A finely drawn capillary pipette, held vertically, will deliver about 50 drops from 1 cc.

Cubic Millimeter.—Unit of space for blood cells. There are 1000 cubic millimeters in 1 cubic centimeter and 1 million cubic millimeters in 1 liter. In water analysis, as there are 1 million milligrams in one liter, parts in the million and milligrams per liter are the same.

1 Meter = 39.37 inches.
1 Centimeter = 0.3937 inch. Approximately, 2/5 inch.
1 Millimeter = 0.0393 inch. Approximately, 1/25 inch.
1 Inch = 25.4 mm.
1 Yard = 0.9144 m.
1 Kilogram = 2.2+ pounds av.
1 Gram = 15.432 grains.
1 Centigram = 0.154 grain.
1 Milligram = 0.0154 grain. Approximately, 1/64 grain.
A pound avoirdupois is equal to 453.59 Gm.
1 Oz. avoirdupois is equal to 28.35 Gm.

One hundred cubic centimeters of a saturated solution contains:

	Water	Alcohol
Methylene blue	6.7	7.0 grams.
Gentian violet	1.5	4.8 grams.
Basic fuchsin	0.66	2.92 grams.

Key to Table on opposite page.

− = negative, + = positive, O = no change, A = acid, Alk. = alkaline, G = gas; Fl = fluorescence, Pep = peptonization. Proteus gives a peculiar dark, heavy oil-like fluorescence in neutral red glucose bouillon.

	Important nonspore-bearing Gram-negative intestinal bacilli	Capsule	Motility	Gelatin liquefaction	Milk (coagulation)	Litmus milk			Glucose (neutral red)	Lactose	Saccharose	Maltose	Mannite	Glucose	Russell's medium		Indol	Voges-Proskauer	Important nonspore-bearing Gram-negative intestinal bacilli
						1 day	3 days	12 days							Butt	Slant			
1.	B. cloacae	−	+	+	+ P	O	A	P	AG Fl	AG	AG	AG	AG	AG	AG	A to Alk	+	+	B. cloacae
2.	B. proteus (x_2; x_{19})	−	+	+	+ P	O	Alk	P	AG Fl	O	AG	O	O	AG	AG	Alk	−	..	B. proteus (x_2; x_{19})
3.	B. coli communis	−	+	−	+	A	A	A	AG	AG	O	AG	AG	AG	AG	A	+	−	B. coli communis
4.	B. coli communior	−	+	−	+	A	A	A	AG	AG	AG	AG	AG	AG	AG	A	+	−	B. coli communior
5.	B. enteritidis (Gaertner)	−	+	−	−	A	Alk	Alk	AG Fl	O	O	AG	AG	AG	AG	A to Alk	−	−	B. enteritidis (Gaertner)
6.	B. paratyphosus A	−	+	−	−	A	A	A to Alk	AG	O	O	AG	AG	AG	AG	Alk	−	−	B. paratyphosus A
7.	B. paratyphosus B	−	+	−	−	A	Alk	Alk	AG Fl	O	O	AG	AG	AG	AG	Alk	−	−	B. paratyphosus B
8.	B. typhosus	−	+	−	−	A	A	A to Alk	A	O	O	A	A	A	A	Alk	−	−	B. typhosus
9.	B. dysenteriae (Flexner-Strong)	−	−	−	−	A	Alk	Alk	A	O	O	A	A	A	A	Alk	±	−	B. dysenteriae (Flexner-Strong)
10.	B. dysenteriae (Shiga-Kruse)	−	−	−	−	A	Alk	Alk	A	O	O	O	O	A	A	Alk	−	−	B. dysenteriae (Shiga-Kruse)
11.	B. dysenteriae (Hiss-Russell; Y)	−	−	−	−	A	Alk	Alk	A	O	O	O	A	A	A	Alk	±	−	B. dysenteriae (Hiss-Russell; Y)
12.	B. morgan No. 1	−	+	−	−	O	O	Alk	AG	O	O	O	O	AG	AG	Alk	+	−	B. morgan No. 1
13.	B. alkaligenes	−	+	−	−	Alk	Alk	Alk	O	O	O	O	O	O	O	Alk	−	−	B. alkaligenes
14.	B. lactis aerogenes	+	−	−	+	A	A	A	AG	AG	AG	AG	AG	AG	AG	A	±	±	B. lactis aerogenes
15.	B. mucosus capsulatus	+	−	−	+	A	A	A	AG	O	AG	AG	AG	AG	AG	A	±	..	B. mucosus capsulatus